Characterizing the potential of DEC-205-mediated antigen delivery to dendritic cells as a tool to induce adaptive immunity against hepatitis C virus infection

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades einer

Doktorin der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von Julia Volckmar

aus Braunschweig

1. Referent: Professor Dr. Stefan Dübel
2. Referentin: Professorin Dr. Dunja Bruder
eingereicht am: 21.09.2011
mündliche Prüfung (Disputation) am: 06.12.2011

Druckjahr 2012

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Tagungsbeiträge

Schiller J., Gereke M., Erck C., Mueller W. and Bruder D. *"Targeting HCV proteins to dendritic cells in vivo to induce antiviral immunity."* **(Poster presentation)**
4th ENII-MUGEN Immunology Summer School 2009, 17-24 May 2009, Capo Caccia, Sardinia (Italy)

Volckmar J., Gereke M., Prettin S., Erck C. and Bruder D. *"Cellular immunotherapy of HCV: Targeting HCV proteins to dendritic cells in vivo."* **(Poster presentation)**
2nd European Congress of Immunology, 13-16 September 2009, Berlin (Germany)

Volckmar J., Gereke M., Prettin S., Erck C. and Bruder D. *"Strategy for vaccination: How to target HCV proteins in vivo?"* **(Poster presentation)**
40th Annual Conference of the German Society of Immunology, 22-25 September 2010, Leipzig (Germany)

Volckmar J., Gereke M., Prettin S., Erck C. and Bruder D. *"Dec-205 on Dendritic Cells: An endocytosis receptor as a target in an efficient HCV vaccination strategy"* **(Poster presentation)**
11th International Symposium on Dendritic Cells in Fundamental and Clinical Immunology, 26-30 September 2010, Lugano (Switzerland)

Table of contents

1 Abstract

Hepatitis C virus (HCV) infection represents a serious worldwide public healthcare problem. Despite extensive investigations no effective vaccine does yet exist. Dendritic cells (DCs) play a pivotal role in mediating immunity to pathogens and represent an exceptionally attractive target for vaccination. Therefore, the aim of this thesis was to test different *in vivo* DC targeting strategies with respect to their applicability for inducing antiviral immunity in the liver and thus to provide the basis for the development of a DC-based HCV vaccine.

In the first part of this thesis two distinct *in vivo* DC targeting strategies, utilizing either the endocytosis receptor DEC-205 or the Toll-like receptor (TLR) 2/6 heterodimer, were compared with respect to their potential to induce widespread cellular and humoral immune responses. After successful chemical conjugation of the model antigen ovalbumin (OVA) to the DEC-205 antibody (αDEC-205), it was shown that αDEC-205/OVA immunization triggered in addition to a vigorous antibody response IFNγ producing $CD4^+$ T cells and cytotoxic T lymphocytes (CTLs). These were, in contrast to all other vaccination strategies tested, also traceable in the liver draining lymph nodes. In line with this, αDEC-205/OVA immunized mice were capable of efficiently clearing the virus-infected hepatocytes. Whereas immunization with αDEC-205/OVA was found to be exceptionally potent in inducing Th1 cells and CTLs, immunization with OVA in addition to the TLR2/6 agonist S-[2,3-bispalmitoyil-oxy-(2R)-propyl]-R-cysteinyl-amido-monomethoxyl poly-ethylene glycol (BPPcysMPEG) resulted in a Th2 dominated $CD4^+$ T cell response and failed to induce CTLs capable of killing virus-infected liver cells. In contrast to BPPcysMPEG-mediated targeting of the full-length OVA protein to DCs, vaccination with BPPcysOVAMPEG which comprises the TLR2/6 agonist linked to the immunodominant MHC-I and MHC-II OVA-peptides, was as efficient as αDEC-205/OVA treatment in inducing antiviral immunity in the liver. However, since vaccination against only a limited number of antigenic determinants is of great disadvantage with respect to highly mutating viruses such as HCV, *in vivo* targeting of antigen to DEC-205 on DCs was identified to be superior to the BPPcysMPEG approach.

In order to extend the analyses towards the design of an effective DC-based HCV vaccine, purification of the recombinant HCV proteins, NS3 and Core, were successfully established followed by chemical crosslinking to αDEC-205. Both conjugates were characterized regarding their capacity to bind to DEC-205. Whereas unrestrained binding of αDEC-205/Core to DCs could be demonstrated, this aspect could not be conclusively clarified for αDEC-205/NS3. However, first immunization trials in mice with both αDEC-205/NS3 and αDEC-205/Core were found to induce HCV-specific immune responses, thus providing a promising starting point for the future development of a DEC-205-based HCV vaccine.

1.1 Zusammenfassung

Hepatitis C Virus (HCV) Infektionen stellen weltweit ein beachtliches gesundheitspolitisches Problem dar. Trotz umfangreicher wissenschaftlicher Studien ist bis heute jedoch kein geeignetes Vakzin verfügbar. Dendritische Zellen (DCs) sind von zentraler Bedeutung für das Auslösen Pathogen-spezifischer Immunität und stellen ein außerordentlich attraktives Target für die Entwicklung von Impfstoffen dar. Ziel dieser Arbeit war es, verschiedene DC *Targeting* Strategien hinsichtlich ihrer Eignung für die Induktion antiviraler Immunität in der Leber zu testen und somit die Grundlage für die Weiterentwicklung eines DC-basierten HCV Vakzins zu schaffen. Im ersten Teil der Arbeit wurden zwei verschiedene Methoden des *in vivo* DC *Targetings* hinsichtlich ihrer Fähigkeit, breitgefächerte zelluläre und humorale Immunantworten auszulösen, charakterisiert. Als molekulare Targets dienten hierfür der Endozytoserezeptor DEC-205 und das TLR2/6 Heterodimer. Vakzinierungsversuche, die nach erfolgreicher chemischer Konjugation des Modellantigens Ovalbumin (OVA) an einen DEC-205-spezifischen Antikörper (αDEC-205) durchgeführt wurden, ergaben, dass αDEC-205/OVA neben einer ausgeprägten Antikörperantwort auch IFNγ produzierende $CD4^+$ T-Zellen und zytotoxische T-Zellen (CTLs) induziert. Im Gegensatz zu allen anderen getesteten Strategien waren die CTL-Antworten in den Leber-drainierenden Lymphknoten αDEC-205/OVA immunisierter Mäuse nachweisbar und in Übereinstimmung damit waren diese Mäuse in der Lage, virale Leberinfektionen zu eliminieren. Im Gegensatz zur der αDEC-205/OVA Immunisierung löste die Vakzinierung mit einer Mischung aus löslichem OVA-Protein und dem TLR2/6 Agonisten BPPcysMPEG eine Th2 dominierte $CD4^+$ T- Zellantwort aus. Zudem wurde keine CTL-Antwort induziert und folglich erfolgte kein CTL-vermitteltes Abtöten virusinfizierter Leberzellen. Neben αDEC-205/OVA führte die Vakzinierung mit BPPcysOVAMPEG, welches ein Konjugat aus dem TLR2/6 Agonisten und den beiden immunodominanten MHC-I und MHC-II OVA-Peptiden ist, ebenfalls zur Induktion antiviraler Immunität in der Leber. Vor dem Hintergrund, dass HCV ein extrem stark mutierendes Virus ist, weist ein Peptidimpfstoff jedoch deutliche Nachteile gegenüber der Vakzinierung mit komplexen Proteinen auf, so dass der DEC-205-basierte Ansatz für die Entwicklung eines HCV Vakzins favorisiert wurde. Hierfür wurden die HCV Proteine NS3 und Core rekombinant hergestellt, aufgereinigt und anschließend an αDEC-205 chemisch gekoppelt. Beide Konjugate wurden umfassend bezüglich ihrer Fähigkeit an DEC-205 zu binden charakterisiert. Eine uneingeschränkte Bindungsfähigkeit konnte für αDEC-205/Core nachgewiesen werden. Initiale Immunisierungsexperimente haben darüber hinaus ergeben, dass sowohl eine αDEC-205/NS3 als auch eine αDEC-205/Core Immunisierung HCV-spezifische Immunantworten induziert. Diese Ergebnisse stellen eine vielversprechende Basis für die zukünftige Weiterentwicklung eines DEC-205-basierten HCV Vakzins dar.

2 Introduction

2.1 The history of vaccination and its impact

The exact time point of vaccine origin or when humans first noticed that they are better at fighting a disease the second time they get it, is unknown. But the early first steps in the history of vaccination date back to the 7th century, when Indian Buddhists drank snake venom to protect themselves from snakebites. The story pursued with first written but unverified records from China about the use of variolation, a process exposing healthy people to live material from the lesions caused by the disease, which was verified by smallpox variolation published in a Chinese medical text in the 18th century (Plotkin & Plotkin 2008). Even though Lady Mary Wortley Montagu (1689-1762) introduced this procedure to Europe as she promoted the technique by effective variolation of her own children against smallpox, the acceptance was generally limited. This was due to deaths caused by the lack of standardization and the resulting perception of variolation as a highly risky procedure (Riedel 2005). In 1798, the English physician Edward Jenner (1749-1823) succeeded an important breakthrough by publishing a report about cowpox as a safe human vaccine for smallpox and called the whole procedure vaccination (latin: *vacca = cow*). Although he was not the first to make use of a relatively non-pathogenic virus to induce immunity against smallpox, Jenner´s work is widely regarded as the foundation of Immunology and was further extended by Louis Pasteur (1822-1895) and others in the following 200 years. This finally peaked with the perhaps greatest success story in vaccination, the eradication of smallpox in 1979, as certified by the World Health Organization (WHO). Moreover, a lot of infectious diseases such as tuberculosis, yellow fever, whooping cough, influenza and tetanus are nowadays largely controlled due to the existence of effective vaccines (Brennan 1998; Plett 2006; Plotkin & Plotkin 2007). Despite these achievements, the demand for vaccine development remains high in the 21st century. This is especially the case, since pathogens such as the HCV or the human immunodeficiency virus (HIV) exist and successfully escape from immune surveillance, by which they establish live-threatening chronic infections for which no effective vaccination strategy could be developed so far. Moreover, emerging and re-emerging diseases like SARS (severe acute respiratory syndrome) and bird/swine flu as well as cancer caused by bacteria, viruses or parasites remain incurable. Taken together, these facts as well as the increase of antibiotic resistances underline the importance of vaccine development and its significance for the health care system (WHO; Chow 1993; Janeway et al. 2001; Taylor et al. 2009; Cavaleiro-Pinto et al. 2011; De Luca & Giraldi 2011). Thus, there is still an urgent need for both new and improved effective vaccines, since

vaccination will remain the most effective tool for preventing infectious diseases and improving public health in the future.

2.2 Vaccination and the role of the immune system

The overall aim of vaccination is to educate the immune system in a manner that facilitates optimal defense and protection against various types of foreign microorganism without disease onset. Thus, detailed knowledge regarding the basic mechanisms underlying pathogen recognition, immune activation and also the existing natural immunological weapons against internal and external threats is of crucial importance for developing effective vaccination strategies.

2.2.1 Innate and adaptive immunity

In mammals, protection against pathogens is mediated through a highly evolved immune system consisting of two distinct but complementary defense mechanisms - innate and adaptive immunity. In face of pathogenic invaders, the immune cells and their effector proteins of both parts of the healthy immune system synergize in an orchestrated and harmonized way. The non-adaptive defense mechanisms act as a first barrier to microbial invaders and moreover play a crucial role in the initiation and the subsequent direction of the adaptive immune response. Different effector cells such as macrophages, DCs, mast cells, neutrophils, eosinophils, natural killer (NK) cells and natural killer T (NKT) cells are activated once a pathogen has crossed the first line of defense, which is comprised of the physical barriers like tight cell-cell contacts, secreted mucus and epithelial cilia. Unlike the unspecific innate host defense mechanisms, the adaptive immune system is composed of B and T cells exhibiting an extremely large diversity of specificities for any individual pathogen. Diversity is achieved through the highly specific T cell and B cell receptors (TCR and BCR, respectively), which develop in vast specificities through clonally unique gene re-arrangements (somatic recombination and somatic hypermutation). Moreover, the adaptive immune system is capable of developing an immunological memory that permits prevention of secondary infections with the same pathogen through establishing a quicker and more effective specific immune response, even decades after the initial sensitizing encounter (Fig. 1) (Janeway et al. 2001; Janeway et al. 2005; Chaplin 2010).

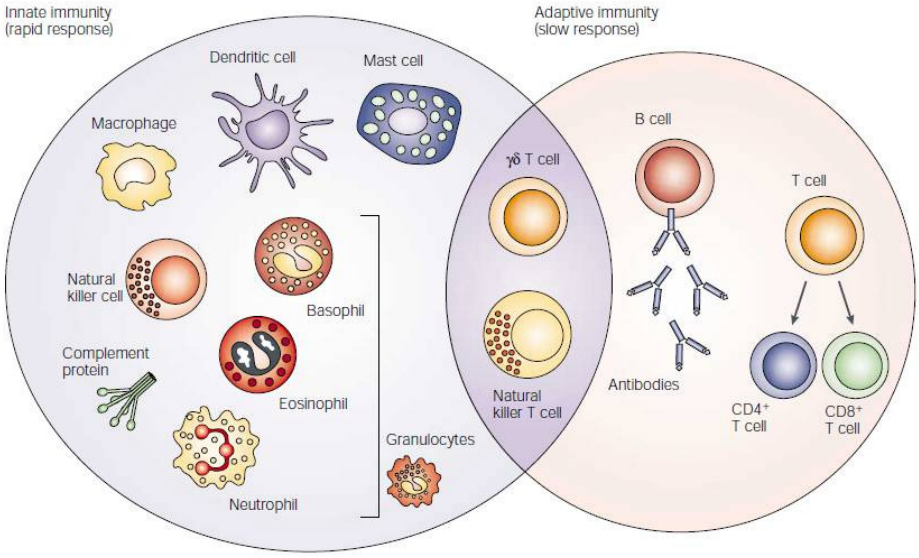

Figure 1. The components of the innate and adaptive immune system.

The components of the innate defense mechanisms play a crucial role in the early phase following pathogen invasion, where a rapid response is essential. In order to fulfill this function, the innate immune system comprises soluble factors such as complement proteins and different effector cells including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer (T) cells. The adaptive defense mechanisms take place 4 to 7 days after first pathogen contact (slow response), but the cellular components (B cells, $CD4^+$ and $CD8^+$ T cells) as well as the humoral part (antibodies) combine more versatile and specific means of defense than the innate response and are additionally able to initiate immunological memory. *Source: Figure was taken from Dranoff 2004.*

2.2.1.1 Pathogen recognition by the cells of the innate immune system

In order to sense potentially harmful microorganisms, effector cells of the innate arm of the immune system express pattern recognition receptors (PRRs). These recognize highly conserved components of pathogenic microorganisms, the so called pathogen associated molecular patterns (PAMPs). Well known examples for PAMPs are bacterial lipopolysaccharide (LPS), unmethylated CpG motifs of bacterial deoxyribonucleic acid (DNA) or viral double-stranded ribonucleic acid (dsRNA) (Janeway 1989; Underhill & Ozinsky 2002; Vasselon & Detmers 2002). These pathogen-derived signals are often referred to as danger signals, since they alert the immune system to the presence of foreign microorganisms (Matzinger 1994; Matzinger 2002). PRRs represent a large group of highly conserved receptor molecules including Toll-like receptors (TLRs), complement receptors, C-type lectin receptors (CLRs) and nucleotide-binding oligomerisation domain (NOD) receptors that can be functionally divided into three classes: secreted, endocytic and signaling PRRs (Medzhitov & Janeway 1997; Gasque 2004; McGreal et al. 2004; Martinon & Tschopp 2005). Whereas secreted PRRs flag microbial components for the complement system and phagocytic cells, the signaling PRRs such as TLRs activate signal-transduction pathways

resulting in the expression of inflammatory cytokines, chemokines and co-stimulatory molecules. The endocytic PRRs like the C-type lectin receptors are expressed on the surface of phagocytes and mediate the uptake of pathogens which subsequently results in the presentation of pathogen-derived antigens to naïve T cells via major histocompatibility complex (MHC) molecules (or human leukocyte antigen (HLA) in human) (Fig. 2). PPRs are expressed on many effector cells of the immune system including macrophages, DCs and B cells as well as on cells that are the first to encounter pathogens during infection, such as surface epithelia. Pathogen recognition via PRRs on the one hand directly induces innate effector mechanisms by stimulating macrophages and neutrophils to act immediately at the sites of pathogen entry. On the other hand, PRRs alert the host organism to the presence of infectious agents and thereby induce adaptive immunity by a variety of endogenous signals. Here, DCs play a pivotal role, since they are not only able to sense infections through PPRs, but are also able to initiate adaptive immune responses (Medzhitov & Janeway 2000; Guermonprez et al. 2002), thereby linking innate and adaptive immunity.

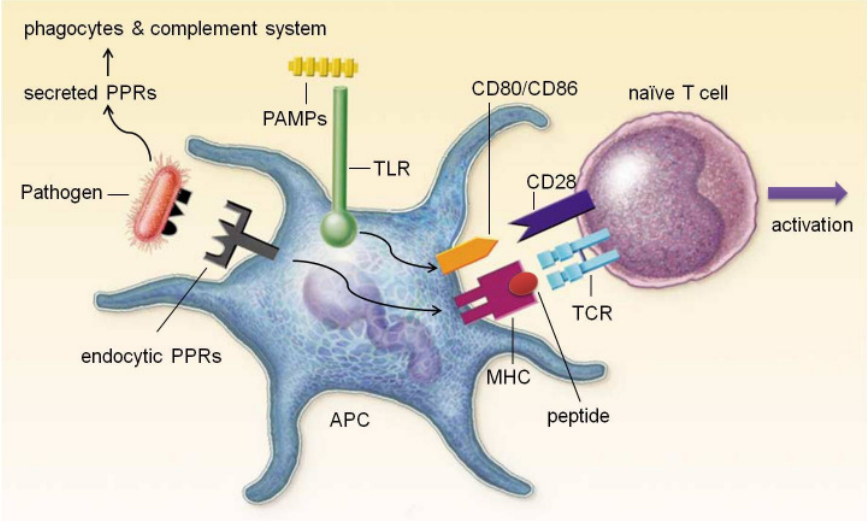

Figure 2. Pattern recognition receptors link the innate and adaptive immune system.

The three classes of pattern recognition receptors (PPRs): the secreted, endocytic and signaling PPRs such as Toll-like receptors (TLRs). See text for detailed description. Abbreviations: Antigen presenting cell (APC); major histocompatibility complex (MHC); pathogen associated molecular patterns (PAMPs); T cell receptor (TCR). *Source: Figure was generated on the basis of Medzhitov & Janeway 2000.*

2.2.1.2 Organization and function of T lymphocytes

As a part of the adaptive immune system, T cells (or T lymphocytes) mediate cellular immune responses and play a key role in the elimination of pathogens. The two major classes of T cells are defined by the expression of the surface molecules CD4 and CD8. $CD4^+$ T cells (helper T cells) display important functions in regulating cellular and humoral immune responses whereas $CD8^+$ T cells (cytotoxic T cells) act to directly kill infected target cells. In order to fulfill these specific tasks, both types of T cells primarily need the aid of additional host cells like professional antigen-presenting cells (APC) such as DCs, which present peptide fragments derived from pathogens on their surface via MHC molecules. Two major classes of MHC molecules, which mainly differ in the source of peptides that they bind and display at the cell surface for T cell recognition, exist. MHC class I molecules (MHC I) are specialized for the presentation of endogenous antigens. They collect peptides derived from cytosolic proteins and are thus able to display fragments of e.g. viral proteins for recognition by the TCR of $CD8^+$ T cells (Fig. 3C). In contrast, $CD4^+$ T cells recognize peptides presented on MHC class II molecules (MHC II), which are derived from exogenous antigens internalized by phagocytic cells (Fig. 3A). However, interactions via the MHC I/TCR or MHC II/TCR complex only deliver a partial signal for T cell activation. Full activation requires the participation of co-stimulatory molecules such as CD28 on the T cell site and CD80/CD86 on the APC site, which altogether initiate a signal cascade in naïve T cells that results in the activation of genes that control lymphocyte proliferation and differentiation. Finally, both naïve $CD8^+$ and $CD4^+$ T cells become activated to differentiate into effector T cells. In the case of $CD8^+$ T lymphocytes, cytolytic proteins including perforin and granzymes are produced and secreted at the point of contact with the target cell, resulting in specific killing of infected cells and subsequent clearance of intracellular pathogens. In addition to cytolysis, $CD8^+$ effector T cells are also able to act via the release of IFNγ and tumor necrosis factor alpha (TNFα) (Fig. 3D). These pro-inflammatory mediators directly influence the function of other cells involved in innate and adaptive immune responses (Janeway et al. 2001; Larosa & Orange 2008; Chaplin 2010). Unlike the cytotoxic activity of $CD8^+$ T cells, the major function of $CD4^+$ T helper cells is the regulation of cellular and humoral immune responses orchestrated through the release of various cytokines and chemokines, which activate and direct other immune cells. Thereby, $CD4^+$ T helper cells can be further divided into different subsets, namely Th1, Th2, Th17 effector cells and regulatory T cells (Treg), each producing an exclusive panel of cytokines. Th1 cells develop from naïve $CD4^+$ T cells under the influence of Inteleukin-12 (IL-12) and IFNγ and possess the capacity to produce IFNγ, TNFα and IL-2. The development of Th2 cells is driven by IL-4 and IL-6 and their specialized role is involved in the humoral part of adaptive immunity. They are producers of IL-4, IL-5, IL-10 as

well as IL-13 and are able to stimulate naïve B lymphocytes to proliferate and differentiate into plasma cells, which in turn secrete antigen-specific antibodies (Fig. 3B). In addition, these two $CD4^+$ T cell subsets can regulate each other. To maintain a balance between both subsets, cytokines from one type of $CD4^+$ T cells inhibit the activation of the other. Thus, IL-10 secreted by Th2 cells inhibits the development of Th1 cells and in turn IFNγ produced by Th1 cells can prevent the activation of Th2 cells (Seder et al. 1994; Janeway et al. 2001; Jelley-Gibbs et al. 2008). Recently, another distinct $CD4^+$ T cell lineage, the Th17 cells, was identified. Since their development seems to be independent from signaling pathways required for Th1 or Th2 cell development, Th17 cells are therefore the product of a distinct $CD4^+$ T cell lineage with unique developmental and functional characteristics. Their survival is supported by the production of IL-6, TGFβ and IL-23 and they are able to produce IL-17, a potent inflammatory cytokine involved in the recruitment and proliferation of neutrophils, as well as IL-17F, IL-6, and TNF. Moreover, Th17 cells are known to play a role in pathogenesis of autoimmune disease (Harrington et al. 2005; Steinman 2007; Jelley-Gibbs et al. 2008). In contrast, the fourth $CD4^+$ T cell subset, the Tregs, act in a clearly opposite way: next to their fundamental function in preventing autoimmunity, they are important for preventing collateral tissue damage and immune pathology caused by an excessive immune response to an acute infection. For these functions, various Treg populations distinguishable by a different cell surface marker profile, by the production of specific cytokines and different modes of action have been identified. The so called naturally occurring $CD4^+CD25^+$ Tregs (nTreg) develop in the thymus and feature immune suppressive capacity to self- and non-self-antigens. In contrast, the induced Tregs (iTreg) originate in the periphery and are mainly identified by their expression of IL-10 and transforming growth factor β (TGFβ). They have been shown to play a role in oral tolerance (Jonuleit & Schmitt 2003).

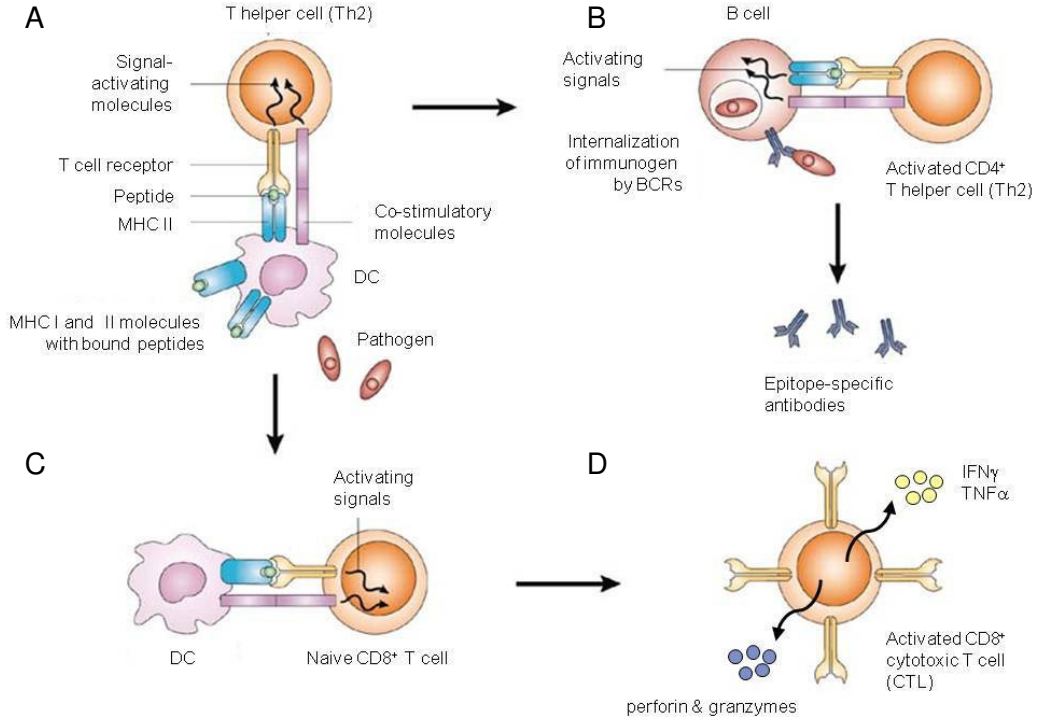

Figure 3. The interplay of dendritic cells, T cells and B cells in immune responses.

The innate and the adaptive immune system are intimately connected with DCs playing a pivotal role as key regulators of T and B cell responses. A) DCs phagocytoze invading pathogens and display pathogen-derived peptides via MHC II on their surface following proteolysis. $CD4^+$ T helper cells interact with DCs through recognition of the peptide/MHC II complexes via their TCR. Additional interactions take place through co-stimulatory molecules that are expressed on DCs (CD80/CD86) and their ligands on $CD4^+$ T cells (CD28). B) These recognition events result in the transmission of activation signals to the $CD4^+$ T helper cell, which is now able to fulfill its task to promote an efficient B cell answer: B cells that display the same peptide/MHC II complexes on their surfaces, acquired as a result of the internalization of the immunogen through their specific surface-immunoglobulin receptors (the BCR), interact with the T helper cell. As a consequence, the B cell is triggered to differentiate into a plasma cell, which is then capable of secreting epitope-specific antibodies. C) DCs are not only able to activate $CD4^+$ T helper cells, but also promote naïve $CD8^+$ T cells to become cytotoxic effector cells, either through peptides derived from immunogens synthesized within the cytosol or by cross-presentation. For this purpose, DCs activate naïve $CD8^+$ T cells through the interaction of the peptide/MHC I complex and the TCR in addition to the co-stimulatory molecules. D) Activated $CD8^+$ CTLs release the cytotoxic proteins perforin and granzymes as well as cytokines such as IFNγ and TNFα. Abbreviations: B cell receptor (BCR); dendritic cells (DCs); naïve $CD4^+$ T helper cells (Th); cluster of differentiation (CD); cytotoxic T lymphocyte (CTL); Interferon gamma (IFNγ); T cell receptor (TCR); tumor necrosis factor alpha (TNFα). *Source: Figure was modified from Purcell et al. 2007.*

2.2.1.3 Function of B lymphocytes and the structure of antibodies

B cells or B lymphocytes, which produce antibodies for pathogen clearance, are the mediators of the humoral part of adaptive immune responses. Activation of naïve B cells can occur with assistance of Th2 effector cells, as mentioned above, or T cell-independently. The T cell-dependent activation results in secretion of the antigen-specific B cell receptor, the membrane anchored Immunoglobulin (Ig) (Fig. 3B). Igs, also referred to as antibodies, are roughly Y-shaped molecules and five different classes can be distinguished based on the constant region of the molecules: IgM, IgD, IgG, IgA and IgE. In general, the specific antibody structure is composed of two heavy (50 kDa) and two light chains (25 kDa), which form a large molecule with a molecular weight of approximately 150 kDa. The two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. The distinctive functional properties of the Ig classes are conferred by the carboxy-terminal part of the heavy chain, which is not associated with the light chain. The specificity of antigen binding is mediated through the amino-terminal sequences of both chains, which vary greatly between different antibodies and are termed variable or V region of the heavy and light chain (V_H and V_L). The remaining domains are constant between immunoglobulin chains of the same isotype, the so called constant or C region of the heavy and light chains (C_H and C_L). Naïve B cells normally express the Ig subtype IgM on their cell surfaces. As a consequence of isotype switching induced by T cell-derived cytokines, matured B cells are able to produce other Ig subtypes such as IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgE and IgA, each with its respective function. Once released, the different isotypes protect from pathogens or pathogen-derived toxins in three different ways: by specific binding to their antigenic epitope they either directly neutralize invaders such as viruses, they mark pathogens for ingestion and destruction by phagocytes, a process known as opsonization, or they are able to activate the complement system. Whereas the first is the simplest and most direct way, all three pathways finally lead to the destruction of pathogenic invaders (Janeway et al. 2001; Chaplin 2010). In most cases, primary infection or vaccination results in prolonged production of high affinity specific antibodies, which provide an important basis for adaptive humoral immunity (Larosa & Orange 2008).

2.2.1.4 Interplay of the innate and adaptive immune system and its importance for vaccination

Considering the various functions of the innate and adaptive immune system, it becomes obvious that both are fundamentally different in their mode of action, but that the synergy between both arms of the immune system is essential for an effective immune response against microbial infections (Chaplin 2010). On the one hand, the adaptive immune response requires signals that provide information about the origin of the antigen and the type of response to be induced, and exactly this information is provided by the innate immune system (Medzhitov and Janeway 1997). On the other hand, the antigen-specific cells of the adaptive immune system enforce their responses by recruiting innate effector cells to ensure complete control of invading pathogens. The finding that the innate and adaptive immune systems are not separated arms of the host defense, but rather intimately connected with each other had great impact on vaccine development (Chaplin 2010). In the past, vaccination approaches mostly focused on the induction of neutralizing antibodies. Current protocols are aiming to implicate other components of the immune system to induce more effective immune responses than does the pathogen itself, which will finally result in an efficient immunotherapy. In this context, DCs have become a fundamental target for developing efficient vaccination strategies. DCs not only represent the key regulators of T cell and B cell responses, but also represent the most important cellular link between innate and adaptive immunity. Thus, these highly professional APCs combine all elements of the immune system needed for designing new generation vaccines (Berzofsky et al. 1999; Berzofsky et al. 2001; Banchereau & Palucka 2005; Tacken et al. 2007).

2.3 Dendritic cells

Ralph Steinman and Zanvil Cohn firstly coined the term DC in 1973 as they described a cell type characterized by its stellate, tree-like shape (Steinman & Cohn 1973). In fact, this observation strongly changed the view of immunologists, since it was realized that this heterogeneous population of APCs initiates T cell-dependent immune responses as efficiently as no other cell type. Moreover, DCs represent the cells connecting the site of pathogen entry with the organs where immune responses take place (Villadangos & Young 2008). For this reason, DCs are regarded as the “police” and the “sentinels” of the immune system (Cools et al. 2007). In addition, they are able to induce two functionally different outcomes when interacting with T cells: tolerance or immunity (Tan & O`Neill 2005). The common model is that DCs in the absence of infections, the so called steady state, present self-antigens to T cells, which in the absence of appropriate co-stimulation leads to apoptosis (Kurts et al. 1997), anergy (Adler et al. 1998) or Treg induction (Verhasselt et al. 2004). Under these conditions, immature DCs are responsible for induction of central tolerance in the thymus as well as for maintaining peripheral tolerance to self-antigens (Brocker et al. 1997; Steinman et al. 2000). In the presence of danger signals derived from microbial invaders, DCs convert from an antigen-sampling mode into a mature, antigen-presenting phenotype associated with a high efficiency to prime T cell clonal expansion into T effector cells, resulting in immunity. Therefore, the interaction of DCs with naïve T cells can lead to different forms of immune responses depending on their activation status (Tan & O`Neill 2005; Cools et al. 2007).

Three signals, which are decisive in determining the fate of naïve T cells following antigen encounter on DCs, have been defined. As a consequence of antigen uptake, the previously immature antigen-capturing DC converts into a mature, antigen-presenting phenotype (Kapsenberg 2003; Villadangos & Heath 2005). In the three signal model, the stimulatory signal 1 resulting from the ligation of the TCR with the peptide-MHC complex on the surface of DCs determines the antigen-specificity of the response (Fig. 4A). Signal 2 is referred to as co-stimulation, which displays an accessory signal mainly mediated through CD28 on T cells and CD80/CD86 expressed on DCs (Fig. 4B). Whereas in the absence of the co-stimulatory signal 2 naïve T cells become anergic or are deleted, the combined action of signal 1 and 2 results in the activation of Tregs, i.e. the outcome is immune tolerance. Efficient induction of immunity in addition requires signal 3, a polarizing signal which determines T cell differentiation into effector cells such as CTLs, Th1 or Th2 cells (Fig. 4C) (Kalinski et al. 1999; Curtsinger et al. 2003). These DC activating or danger signals consist of pro-inflammatory cytokines and PAMPs, which induce high-level expression of selective cytokines or membrane-bound factors such as IL-12 and CC-chemokine ligand-2 (CCL-2).

Thus, the nature of the T cell polarizing signal 3 and therefore the outcome of the immune response strongly depend on the nature of the danger signals sensed by DCs (Trinchieri 2003; Kapsenberg 2003; Macagno et al. 2007).

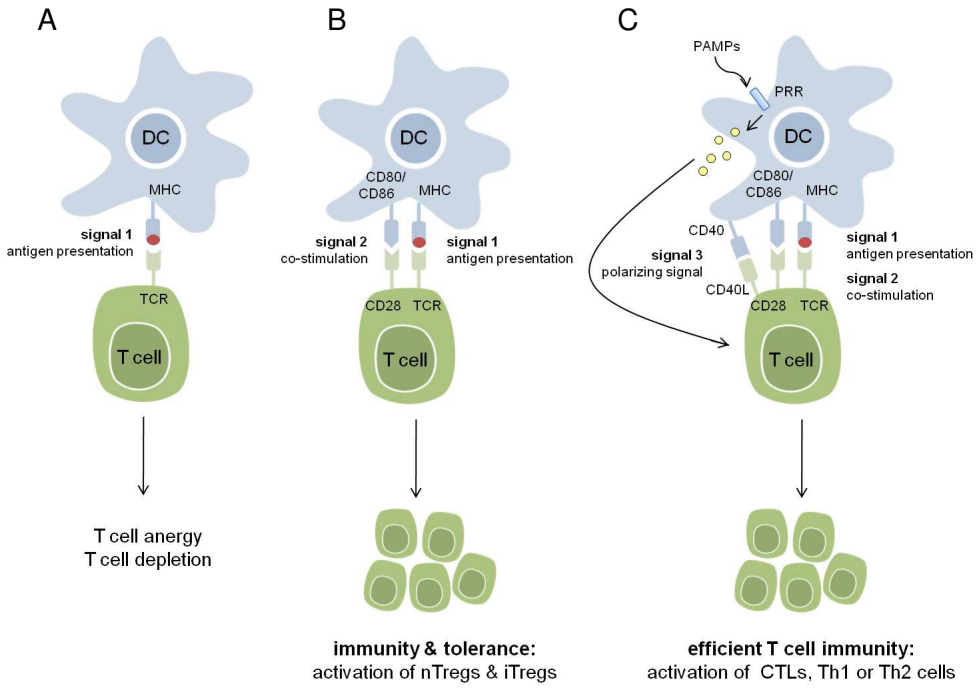

Figure 4. A simplified view of the 3 signal model: induction of tolerance or immunity by dendritic cells.

DCs deliver three different signals that are thought to determine the fate of naïve T cells. See text for detailed description. Abbreviations: dendritic cells (DCs); major histocompatibility complex (MHC); pathogen associated molecular patterns (PAMPs); pattern-recognition receptors (PRR); T cell receptor (TCR); inducible regulatory T cells (iTreg); naturally occurring regulatory T cells (nTregs). *Source: Figure was generated on the basis of Kapsenberg 2003 and Cools et al. 2007.*

Since DCs have become a promising target for immunotherapy, in depth understanding of the exact mechanisms how these cells act to prime T cells are of crucial importance to manipulate the immune system in the desired manner. In this context, the multifaceted function of DCs to either induce tolerance or immunity, have to be considered for defining the experimental settings suitable for the respective DC-based therapeutic approach. As mentioned above, PAMPs, TLRs and T cell polarizing factors directly influence the outcome of DC-mediated immune responses. Therefore, the choice of the particular adjuvant either provoking tolerance or protective immunity is decisive for the outcome of vaccination. Moreover, the function of DCs differs between the various subsets described below, so that the consideration of targeting only a specific group of DCs or as many subsets as possible should be included for designing effective vaccines. Nevertheless, the targeted manipulation of DCs opens up the unique possibility for versatile immunotherapeutic strategies, not given for any other cell type in the immune system. Thus, DC-based therapies have been

successfully used to either enhance immunity toward persistent pathogens and tumors as well as to down-modulate excessive immune responses in the context of allergy, autoimmunity and transplantation (Figdor et al. 2004; Pulendran 2005; Steinman & Banchereau 2007; Cools et al. 2007; Delamarre & Mellman 2011).

The pivotal role of DCs in linking innate and adaptive immunity underlines that they represent key players of the immune system with outstanding therapeutic potential. Intensive investigations during the last decade revealed that the multifaceted functions of DCs are not achieved by a single cell type, but rather by a pool of different DC subsets exhibiting an extremely high phenotypic plasticity (Shortman & Liu 2002: Cools et al. 2007; Shortman & Naik 2007).

2.3.1 Dendritic cell subsets and their development

DCs consist of a network of multiple subtypes, which differ in their location, migratory capacity, function and dependence on inflammatory stimuli needed for their development (Shortman & Naik 2007). Of note, some subsets found in mice cannot directly be transferred to humans, since they may differ in the expression of several surface molecules such as CD8, the major marker used to segregate mouse conventional DC (cDC) subsets, is not expressed by human cDCs (Shortman & Liu 2002). Nevertheless, all DC subsets are derived from multipotent hematopoietic stem cells (HSC) in the bone marrow. In mice, all blood and tissue DCs develop from the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) through FMS-related tyrosine kinase 3 ligand ($Flt3^+$) progenitors (Manz et al. 2001; Colonna et al. 2006). DCs can be divided into two major groups, the cDC, which already display the typical DC morphology and exhibit DC function in the steady state, and precursors of DCs (pre-DC), which require further development to acquire the typical DC morphology and full DC function. Both monocytes and plasmacytoid DCs (pDC) belong to the family of pre-DC, which during this intermediate stage are mainly found in the blood (Shortman & Naik 2007). As a consequence of infection or inflammation these pre-DCs can mature into a monocyte-derived inflammatory DC or activated pDC and migrate to the draining lymph node, where they present the captured antigen to T cells (Grouard et al. 1997; Randolph et al. 1999; Liu 2005). The second group of cDCs can be separated into migratory DCs and lymphoid-tissue resident DCs. Dermal DCs, interstitial DCs (IDC) and Langerhans DCs (LDC) belong to the subfamily of migratory DCs. While dermal DCs are most often identified in the dermis, LDCs are the only DCs found in the epidermis, but also in the epithelia of the intestinal, respiratory and reproductive tracts. They are moreover distinguished by their functions, since dermal DCs initiate T cell responses, whereas LDCs suppress them (Shortman & Naik 2007; Kaplan 2010). The group of migratory DCs are regarded as the classical text-book DCs (Bell et al. 1999), since they act as antigen-sampling

sentinels in the periphery and migrate to the lymph nodes to present peripheral antigens when activated and matured, but also constitutively. In contrast, the function and presence of lymphoid-tissue resident cDCs is restricted to one lymphoid organ such as spleen and thymus, i.e. they do not migrate through the lymph and most notably collect and present foreign and self-antigens in the tissue itself. This subtype can be also separated in DCs, which either express high levels of $CD8^+$ ($CD4^-CD8^+$) or that lack this marker ($CD4^-CD8^-$; $CD4^+CD8^-$) (Ardavin 1997; Vremec et al. 2000; Shortman & Naik 2007). The $CD8^+$ and $CD8^-$ cDCs differ in their immune functions and the expression of specific surface markers such as the C-type lectin DEC-205. While $CD8^+$ cDCs preferentially activate cytotoxic T cells, the $CD8^-$ cDCs selectively stimulate $CD4^+$ T cells, both accompanied by a different cytokine production profile and the ability to cross-present antigens (Hochrein et al. 2001; Pooley et al. 2001; Colonna et al. 2006). Moreover, the $CD8^+$ DC population is $DEC\text{-}205^+CD11b^-$, in contrast to the second population, the $CD8^-$ that is $DEC\text{-}205^-CD11b^+$ (Fig. 5A) (Vremec & Shortman 1997).

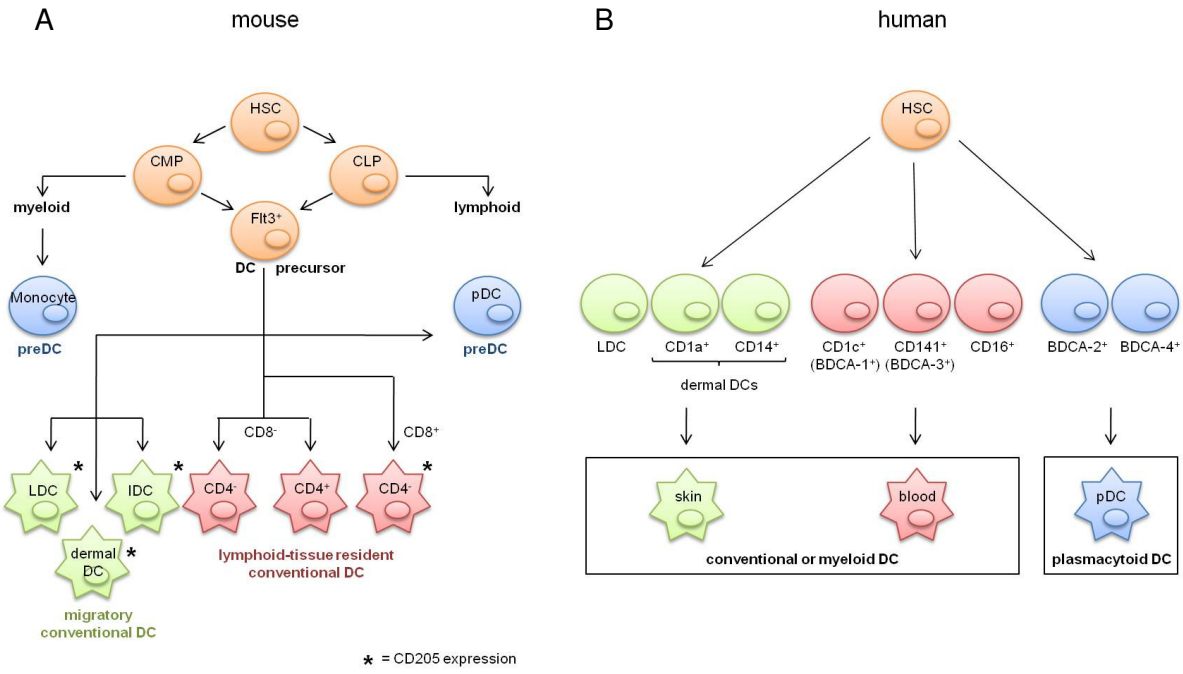

Figure 5. Model of mouse and human dendritic cell subsets and their development.

Differentiation steps of murine (A) and human (B) DCs in the steady state. In mice, the endocytosis receptor CD205 (*) is mainly expressed by $CD8^+CD4^-$ cDCs, but also by LDC and dermal DCs. In humans, it is not restricted to a specific cell type, but rather expressed by many different cell types. See text for detailed description. Abbreviations: blood dendritic cell antigen (BDCA); conventional DC (cDC); common myeloid progenitor (CMP); common lymphoid progenitor (CLP); dendritic cells (DCs); FMS-related tyrosine kinase 3 ligand (Flt3+); hematopoietic stem cell (HSC); interstitial DCs (IDC); Langerhans dendritic cells (LDC); myeloid dendritic cell (mDC); precursors of DCs (pre-DC). *Source: Figure was generated on the basis of Colonna et al. 2006, Shortman & Naik 2007; Piccioli et al. 2007 and Delamarre & Mellman 2011.*

In humans, the multiple DC subsets are as well derived from the hematopoietic stem cells and develop into different DC precursors present in the blood and the skin (Shortman & Liu 2002; Woltman et al. 2010). They are characterized by the heterogeneous expression of a wide range of markers as well as in their response to pathogens, their antigen processing capacity and their potential to activate T cells. However, in contrast to mice, human DCs consist of fewer sub-lineages, but differ to a larger extent in their maturation states (Dudziak et al. 2007; Hart 1997; Kadowaki et al. 2001). Fully differentiated human DCs can be divided into the two major subsets: pDCs and cDCs or mDCs, respectively. The rare subset of pDCs (0.3 - 0.5 % of the human peripheral blood) can be distinguished by specific surface markers such as the blood dendritic cell antigen (BDCA)-2$^+$ or BDCA-4$^+$. They are considered to play an important role in viral defence, since they produce high amounts of type I interferons (IFN) such as IFNα and IFNβ. Although the capacity of these DCs to prime productive T cell responses after infection or immunization is well documented, an opposite tolerogenic role for pDCs has also been proposed. The cDCs can also be divided into multiple subtypes based on the presence either in the skin or blood as well as on the expression of specific surface markers (Cella et al. 1999; Siegal et al. 1999; Ito et al. 2007; Delamarre & Mellman 2011; Reizis et al. 2011). Thereby, three cDC subsets exist in the skin: the CD14$^+$ and CD1a$^+$ dermal DCs as well as the LDCs in the epidermis, each with distinct functions. While LDCs are superior at cross-presentation, CD14$^+$ DCs preferentially prime humoral immunity and CD1a$^+$ DCs could activate CD8$^+$ T cells better than CD14$^+$ DCs, but less efficiently than LDCs (Klechevsky et al. 2008). The human blood cDCs consists of CD1c$^+$ (BDCA-1$^+$), CD141$^+$ (BDCA3$^+$) and CD16$^+$ DCs (Dzionek et al. 2000; MacDonald et al. 2002; Piccioli et al. 2007). The CD141$^+$ (BDCA3$^+$) DCs, which are highly efficient in cross-presentation, and the CD1c$^+$ (BDCA-1$^+$) DCs, which have been shown to be more efficient at antigen presentation on MHC-II, were suggested to be the equivalent to murine CD8$^+$ and CD8$^-$ DCs, respectively (Fig. 5B) (Robbins et al. 2008; Bachem et al. 2010; Delamarre & Mellman 2011).

2.3.2 Antigen uptake, processing and presentation by dendritic cells

As sentinels of the immune system, immature DCs permanently patrol through the blood, peripheral tissues, lymph and secondary lymphoid organs to capture and probe all kinds of antigens such as harmless self-antigens, apoptotic tissue cells as well as pathogenic and nonpathogenic foreign antigens. In order to do so, they exhibit high endocytic capacity, constitutively taking up antigen by macropinocytosis, phagocytosis and receptor-mediated endocytosis (Steinman & Swanson 1995; Guermonprez et al. 2002). Macropinocytosis is a cytoskeleton dependent type of fluid-phase antigen uptake and occurs constitutively in non-activated DCs. Therefore, it represents a critical antigen uptake mechanism which enables DCs to sample large amounts of surrounding fluids rapidly and nonspecifically. In contrast,

large antigenic particles are ingested by phagocytosis. By means of the receptor-mediated endocytosis or so called clathrin-dependent endocytosis, proteins are taken up by specific receptors expressed on the surface of DCs. Proteins that enter the cell by receptor-mediated endocytosis are cleared from the surface via clathrin-coated pits that mediate the transport to the early and late endosome for antigen processing (Fig. 6) (Sallusto et al. 1995; Mellman 1996; Mukherjee et al. 1997; East & Isacke 2002).

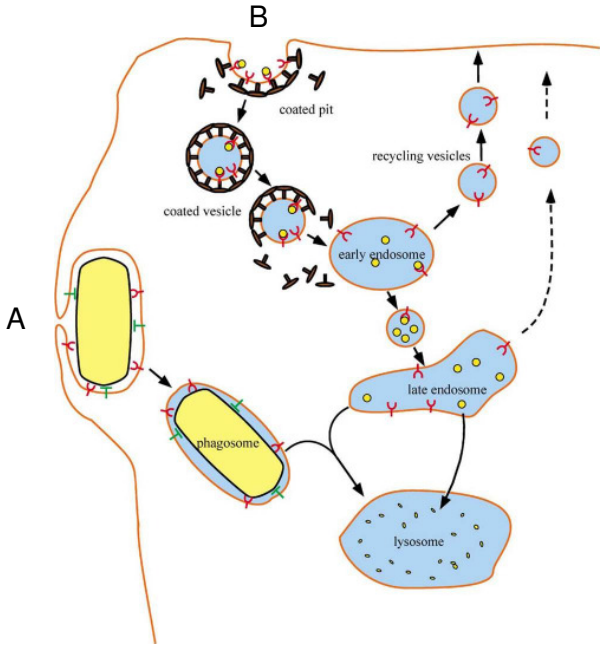

Figure 6. Phagocytosis and receptor-mediated endocytosis.

Antigens can be internalized by either phagocytosis (A) or receptor-mediated endocytosis (B). A) As a consequence of phagocytosis, antigens are internalized by actin-dependent membrane movement to engulf the phagocytozed particles. The resulting phagosome subsequently forms a degradative phagolysosome. B) Receptor-mediated endocytosis is mediated by different receptors such as the C-type I lectin DEC-205 and the macrophage mannose receptor (MMR). The antigen/receptor complex is rapidly internalized via clathrin-coated vesicles and delivered to either the early (MMR) or late endosomes (DEC-205), from where the receptors are able to recycle to the cell surface. *Source: Figure was taken from East & Isacke 2002.*

2.3.2.1 Antigen processing and (cross-)presentation following receptor-mediated endocytosis

After internalization of the antigen/receptor complexes via coated pits, the macromolecules are transported to the early/late endosomes and lysosomes. Most of the antigens remain in the endosomes where they are degraded into smaller peptides after the fusion of endosomes with protease containing lysosomes (Luzio et al. 2009; Pandey 2009). These antigenic peptides are subsequently loaded on newly synthesized MHC-II molecules and the peptide/MHC-II complexes are transported to the cell surface for presentation to $CD4^+$ T cells (Watts 1997; Turley et al. 2000). Some DCs, such as the $CD8^+CD4^-$ cDCs, exhibit the unique

capability to present extracellular antigens on MHC-I molecules, a process termed cross-presentation (Pooley et al. 2001). Here, small quantities of internalized antigen escape from the endosomes to the cytosol where they are further degraded by the proteasomes, followed by transported to the ER, where they are loaded on newly synthesized MHC-I molecules and finally presented to $CD8^+$ T cells on the DC surface (Yewdell et al. 1999; Heath et al. 2004; Cresswell et al. 2005). Although to date, the exact circumstances that promote cross-presentation remain largely unclear, the capability of DCs to cross-present antigens generally enables the induction of cytotoxic T cell responses against extracellular pathogens, which would not infect professional APCs. Of note, cross-presentation of exogenous antigens will not only induce priming of pathogen-specific CTLs, but also induce cross-tolerance by constitutive presentation of self-antigens to cause deletion of self-reactive CTLs (Kurts et al. 1998; Lin et al. 2008). Various receptors have been shown to confer the ability to enhance cross-presentation by DCs including the C-type lectin receptors (Bozzacco et al. 2010).

2.3.2.2 C-type lectin receptors

Specialized subsets of DCs which are capable of receptor-mediated endocytosis express a large diversity of endocytosis receptors, which upon binding induce the internalization of antigen (Guermonprez et al. 2002). The endocytosis receptor family comprises the Fc-receptor (FcR), which is able to bind the Fc domain of immunoglobulins, specific receptors for heat shock proteins and scavenger receptors (Fanger et al. 1996; Arnold-Schild et al. 1999; Castellino et al. 2000; Shakushiro et al. 2004). Next to these, one group of particular importance is represented by the family of C-type lectins, which are either produced as transmembrane proteins or as soluble receptors such as the lung surfactant proteins A and D (Wintergerst et al. 1989; Figdor et al. 2002). Soluble CLRs function by ligation and opsonization of microorganisms, whereas membrane-bound CLRs are designed to capture antigens for intracellular destruction, degradation and loading on MHC molecules. Two groups of membrane-bound CLRs exist, the C-type I and II lectins, which can be distinguished on the basis of their molecular structure (Figdor et al. 2002). DCs are able to express the C-type I lectin DEC-205 (CD205) (Jiang et al. 1995) and the macrophage mannose receptor (MMR) (CD206) (Sallusto et al. 1995) as well as several C-type II lectins including Langerin (CD207) (Valladeau et al. 2001; Valladeau et al. 2002), DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) (CD209) (Geijtenbeek, Torensma et al. 2000) and BDCA-2 (Dzionek et al. 2001). Several of the membrane-bound C-type lectins function as endocytic receptors. DEC-205 and MMR for example share a characteristic property: they are rapidly internalized from the plasma membrane for the delivery of bound material into the endosomal system via clathrin-coated vesicles and the receptor is subsequent recycled to the cell surface (Fig. 6). In general, the binding of a specific ligand to CLRs occurs in a

calcium-dependent manner (East & Isacke 2002; Figdor et al. 2002). The so far identified ligands binding to CLRs represent either endogenous self-molecules, which mediate cell-cell interactions during immune responses, or pathogens and pathogen-derived ligands such as gp120 from HIV (McGreal et al. 2005). Thus, although generally displaying important immunological functions, pathogens can misuse DC endocytic receptors for their own benefit, especially those receptors that facilitate the migratory capacity of DCs such as DC-SIGN, which captures HIV-1 in the periphery and facilitates its transport to secondary lymphoid organs rich in T cells to enhance infection in trans of these target cells (Tab. 1) (Geijtenbeek, Kwon et al. 2000; Guermonprez et al. 2002).

C-type lectin	Type	Production	Ligands (selected)	Function
DEC-205 (CD205)	I	DCs, LCs, high on activated DCs, thymic ECs	?	antigen uptake
MMR (CD206)	I	DCs, LCs, monocytes, macrophages	mannose fucose	antigen uptake
Langerin (CD207)	II	LCs	mannose fucose	formation of birbeck granules
DC-SIGN (CD209)	II	DCs	HIV (gp120) other pathogens ICAM-2 and -3	T cell interaction migration antigen uptake
BDCA-2	II	pDCs	?	antigen uptake?
DCIR-2	II	DCs, monocytes, macrophages	?	?
Dectin 1	II	DCs, LCs	β-glucan	T cell interaction
Dectin 2	II	DCs, LCs	?	antigen uptake

Table 1. Characteristics of C-type lectins expressed by dendritic cells.

The table summarizes the most important C-type lectins expressed by DCs and used for *in vivo* targeting of antigen to DCs. Abbreviations: blood dendritic cell antigen-2 (BDCA-2); dendritic cells (DCs); DC inhibitory receptor 2 (DCIR-2); DC-specific ICAM-3 grabbing non-integrin (DC-SIGN); intercellular adhesion molecule-2 and -3 (ICAM-2 and -3) Langerhans cells (LCs); macrophage mannose receptor (MMR); thymic endothelial cells (thymic ECs). *Source: Table was adapted from Figdor et al. 2002 and McGreal et al. 2005.*

The expression of the C-type lectins differs between DC subsets depending on their activation state and tissue localization (Figdor et al. 2002). For example mouse splenic $CD8^+$ DCs express high levels of DEC-205, whereas the $CD8^-$ ones are considered to be $DEC\text{-}205^-$ but DC inhibitory receptor 2^+ (DCIR-2) (Dudziak et al. 2007). Since C-type lectin receptors have gained great importance in particular with regard to the development of new therapeutic approaches based on the *in vivo* targeting of antigen to DCs, the CLR expression diversity

within the different DC subsets has to be considered for each respective therapeutic application. Moreover, there are differences in the cell-type-specific expression of CLRs between humans and mice. For instance, whereas DEC-205 expression is largely restricted to DCs in mice, its expression is far less restricted to DCs in humans (Inaba et al. 1995; Kato et al. 2006; Tacken et al. 2007).

2.3.2.3 Toll-like receptors

Another important group of receptors expressed by DCs are the non-phagocytic TLRs, which respond to a wide variety of pathogen-derived danger signals or PAMPs (2.2.1.1). So far, 13 different mammalian TLRs have been identified, 10 in humans and 13 in mice, each expressed in the distinct cellular compartment (Beutler 2009; Shi et al. 2011). Whereas TLR1, 2, 4, 5, 6, 10 and 11 (only present in mice) are expressed at the cell surface and can be activated by molecules generally located at the surface of bacteria, fungi, or protozoa (Hoshino et al. 1999; Hayashi et al. 2001; Takeuchi et al. 2001; Zhang et al. 2004; Hasan et al. 2005; Jin et al. 2007), TLR 3, 7, 8, and 9 are located in the endoplasmic reticulum and recognize bacterial or viral nucleic acids (Tab. 2) (Bell et al. 2005; Haas et al. 2008). Ligand binding initiates a complex signaling cascade, which finally leads to the induction of early immune responses. Following ligand-binding by a TLR, one of two pathways is triggered, depending on the specific TLR. This is either the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway resulting in the production of pro-inflammatory cytokines or the MyD88-independent pathway, which is associated with IFNβ responses (Mukhopadhyay et al. 2004; Takeda & Akira 2004). In order to bind their specific ligands and to initiate downstream signaling pathways, certain TLRs have to heterodimerize (TLR2/TLR1; TLR2/TLR6) or homodimerize (TLR3/TLR3) (Ozinsky et al. 2000; Takeuchi et al. 2001; Takeuchi et al. 2002; Leonard et al. 2008). The heterodimer TLR2/TLR6 senses di-acylated lipopeptides, for example the macrophage-activating lipopeptide-2 (MALP-2) (Takeuchi et al. 2001). TLR3 ligands comprise viral single-stranded (ss) and dsRNA released by damaged or dying cells during infection as well as the synthetic viral dsRNA analog polyinosine-polycytidylic acid Poly (I:C) (Alexopoulou et al. 2001; Karikó et al. 2004). Interestingly, TLR3 signaling is known for its Th1 polarizing potential, so that it plays an important role in biasing responses to viral infections (Vercammen et al. 2008). Since TLR expression differs within the various DC subsets, the TLR expression pattern is regarded to define the nature of the immune response induced following infection. In humans, TLR3 is expressed in cDCs, but absent from pDCs. In contrast, the TLR expression by DCs is less restricted in mice, with the only exception being TLR7 which is not expressed by $CD8^+$ splenic cDCs (Muzio et al. 2000; Kadowaki et al. 2001; Edwards et al. 2003; Zanoni & Granucci 2010; Khoo et al. 2011). Moreover, it has been demonstrated that TLRs collaborate with CLRs, such as TLR2 with

Dectin-1. This collaboration induces cumulative effects of physical or functional interactions that further direct the outcome of the immune response (Mukhopadhyay et al. 2004).

TLR	location	Selected ligands	Origin of ligand
1/2	plasma membrane (cell surface)	triacyl lipopeptides	bacteria & mycobacteria
2	plasma membrane (cell surface)	lipoprotein/lipopeptides peptidoglycan Heat-shock protein 70	various pathogens gram-positive bacteria host
3	endosome	ssRNA dsRNA **Poly (I:C)**	West Nile virus Reovirus **synthetic dsRNA**
4	plasma membrane (cell surface)	LPS Mannan heat-shock protein 70	gram-negative bacteria candida host
5	plasma membrane (cell surface)	flaggelin	bacteria
6/2	plasma membrane (cell surface)	diacyl lipopeptides lipoteichoic acid **BPPcysMPEG***	mycoplasma gram-positive bacteria **synthetic derivative of MALP-2**
7	endosome	ssRNA	viruses (Influenza virus)
8	endosome	ssRNA	RNA virus
9	endosome	dsRNA **CpG containing DNA**	murine cytomegalovirus **bacteria & viruses**
10	plasma membrane (cell surface)	?	?
11	plasma membrane (cell surface)	Profilin	*Toxoplasma gondii* uropathogenic bacteria
12	unknown	?	?
13	endosome	?	virus

Table 2. Human and murine Toll-like receptors and their ligands.

The human and murine TLRs are summarized together with their natural or synthetic ligands. TLR8 refers to humans, whereas TLR11, 12 and 13 have so far only been identified in mice. The highlighted ligands represent the TLR agonists used in this thesis. *BPPcysMPEG = S-[2,3-bispalmitoyiloxy-(2R)-propyl]-R-cysteinyl-amido-monomethoxyl poly-ethylene glycol (Prajeeth et al. 2010). Abbreviations: deoxyribonucleic acid (DNA); lipopolysaccharide (LPS); mycoplasma macrophage activating lipopeptide-2 (MALP-2); polyinosine:polycytadilic acid (Poly (I:C)); double-stranded ribonucleic acid (dsRNA); single-stranded ribonucleic acid (ssRNA); Toll-like receptor (TLR). *Source: Table was modified according to Akira & Takeda 2004; Kumar et al. 2009; Beutler 2009; Shi et al. 2011.*

The stimulation of a TLR by binding to its specific ligand induces DC maturation (2.3.). This is associated with increased efficiency in antigen processing and presentation via MHC molecules as well as up-regulation of co-stimulatory molecules. These mature DCs are extremely potent in activating naïve T cells. Moreover, upon maturation, additional changes

in DC phenotype and function occur, including decreased endocytic capacity, cytoskeleton rearrangement, increased migration as well as survival and death depending on the type of DC analyzed (Zanoni & Granucci 2010). Due to their unique immune-activating properties, TLR agonists represent promising candidates for the development of improved vaccine adjuvants (Duthie et al. 2011). In line with this, several different *in vivo* DC targeting studies revealed that the combined application of specific TLR agonists such as Poly (I:C) (TLR3) and/or CpG (TLR9) together with the respective antigen induces strong and protective T cell responses in different experimental settings and provide synergistic effects on T cell activation (Bonifaz et al. 2004; Mahnke et al. 2005; Boscardin et al. 2006; Trumpfheller et al. 2006; Johnson et al. 2008; Trumpfheller et al. 2008; Zhu et al. 2008; Zhu et al. 2010).

2.3.3 Dendritic cell-based immunotherapy

Since DCs have been identified as key players in orchestrating innate and adaptive immune responses, they are in focus as potential targets for immunotherapy and thus several strategies have been investigated regarding their potential use in vaccine development (Caminschi 2009). In recent years, techniques for the isolation of large numbers of DCs *in vitro*, either derived from monocytes or from $CD34^+$ hematopoietic precursors that are isolated from patient blood, were developed for use in clinical trials (Tacken et al. 2007). Meanwhile, various protocols such as peptide loading, RNA/DNA transfection, viral gene transfer and viral infection have been designed for *ex vivo* loading of antigens onto DCs. The most commonly used protocol is based on the *in vitro* loading of DCs with MHC-I or MHC-II (HLA-I and HLA-II in humans) binding antigenic peptides. These antigen-pulsed DCs are cultured in the presence of various cytokines and maturation stimuli followed by intravenous (i.v), intradermal or intranodal administration to the patient. Nevertheless, *in vitro* peptide pulsing is regarded as suboptimal, since this technique requires identification of relevant peptide sequences for each antigen on the basis of the MHC haplotype of every individual patient. Moreover, a further disadvantage of DC loading with MHC I peptides is the lack of $CD4^+$ T cell help which would support the efficient generation of antigen-specific cytotoxic T cells as well as B cell responses. To circumvent these problems, longer peptides including both MHC-I and MHC-II epitopes or DC loading with whole proteins seem to be more promising (Takahashi H. et al. 1993; Figdor et al. 2004; Tuyaerts et al. 2007). Another possibility is the genetic modification of DCs in order to overcome problems of peptide/protein loading. *In vitro* genetic modification of DCs can be achieved either by non-viral delivery systems such as transfection (Yang et al. 1999; Mitchell & Nair 2000) or electroporation (Van Tendeloo et al. 1998; Van Tendeloo et al. 2001) of desired naked DNA/RNA constructs, or by viral vectors encoding target sequences based on pox viruses, herpes simplex viruses (HSVs), adenoviruses, adeno-associated viruses (AAVs),

retroviruses and lentiviruses (Breckpot et al. 2004). The advantage of both strategies is that a combination of MHC-I and MHC-II restricted epitopes can be incorporated to obtain diverse antigen presentation (Tuyaerts et al. 2007). Nevertheless, the major disadvantages of current DC-based therapies that are based on *in vitro* expansion and/or manipulation of autologous DCs are that they are cost-intensive, have to be custom-made for each individual and are limited to specific DC subsets. A promising alternative approach of wider application displays the targeting of antigens to DCs *in vivo*, which gained a lot of research interest in the past decades (Fig. 7) (Tacken & Figdor 2011).

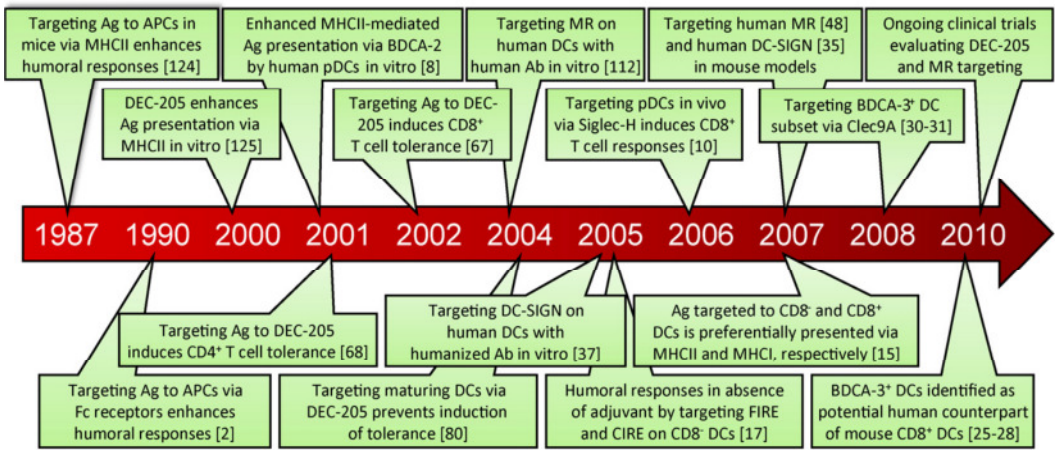

Figure 7. Progress made in *in vivo* targeting of antigen to dendritic cells.

The figure summarizes selected studies concerning antigen targeting to DCs *in vivo* of the past three decades. Abbreviations: antibody (Ab); antigen (Ag); antigen-presenting cell (APC); blood dendritic cell antigen (BDCA); C-type lectin receptor 9A (CLEC-9A); dendritic cell (DC); DC-specific ICAM-3 grabbing non-integrin (DC-SIGN); F4/80-like receptor (FIRE); mannose receptor (MR); major histocompatibility complex (MHC); plasmacytoid dendritic cells (pDC); *Source: Figure was taken from Tacken & Figdor 2011.*

Various useful receptors have been identified for targeting DCs *in vivo,* especially those mediating endocytosis (Tacken & Figdor 2011). Next to others, Snider and colleagues opened the field of *in vivo* DC-based immunotherapy as they used heterocrosslinked bi-specific antibodies recognizing both the selected antigen and the FcR on the surface of DCs, which led to enhanced immunogenicity to the particular antigen (Snider et al., 1990). Besides the FcRs, further endocytosis receptors more specifically expressed by DCs were identified, first of all the family of C-type lectin receptors that have gained increasing importance for vaccination strategies (2.3.2.2) (Tacken et al. 2007). In order to target DCs *in vivo*, constructs consisting of an antibody with high specificity and affinity for the respective endocytosis receptor MMR (Keler et al. 2004; Ramakrishna et al. 2004; He et al. 2007), DEC-205 (2.3.3.1) (Bonifaz et al. 2002; Mahnke et al. 2003; Bonifaz et al. 2004) or DC-SIGN (Pereira et al. 2007) fused to a selected antigen against which the immune response was to be induced were generated. These *in vivo* DC targeting strategies were shown to induce

exceptionally effective adaptive immune responses (Tacken et al. 2007). In this thesis special focus was placed on the endocytosis receptor DEC-205.

2.3.3.1 DEC-205 targeting

DEC-205, a 205 kDa protein also known as CD205, gp200-MR6 or LY75 (lymphocyte antigen 75) (Butler et al. 2007) is a C-type lectin I receptor (2.3.2.2) that has been identified as the antigen recognized by the monoclonal antibody (mAb) NLDC-145 on mouse DCs. This antibody distinguishes non-lymphoid DCs (NLDC) from bone marrow precursor cells and macrophages (Kraal et al. 1986). Approximately ten years after its discovery, Jiang et al. and Witmer-Pack et al. observed that following maturation, the DEC-205 expression on the surface of DCs increases and that DEC-205 features endocytic properties. This led to the suggestion that DEC-205 functions as a receptor mediating antigen uptake and subsequent targeting to intracellular compartments (Jiang et al. 1995; Witmer-Pack et al. 1995). Since its original identification, DEC-205 function has been analyzed in more detail. Not only the mechanisms of its endocytic properties were discovered, but also additional specific properties of DEC-205 could be identified.

To serve its endocytic function, DEC-205 possesses a specific structure, which clearly differs from MMR and the C-type II lectins. The extracellular domain of DEC-205 contains a cysteine-rich domain (CR), a fibronectin type II repeat (FN) and ten carbohydrate recognition domains (CRD), but none of its ten CRD-like domains seem to contain the consensus amino acid sequences required for calcium or carbohydrate binding (Jiang et al. 1995; McKay et al. 1998; Figdor et al. 2002). In contrast, the relatively short cytoplasmic tail contains a tyrosine-based motif for internalization in clathrin-coated vesicles and a distinct distal region with a triad of acidic amino acids, referred to as EDE (Fig. 8). This region mediates the efficient transportation of endocytosed DEC-205 into more central regions of the cell, more specifically, to the late endosomes and the MHC II compartments, which is followed by recycling of the intact receptor to the cell surface (Fig. 6). Its targeting to the late endosomes displays a specific and unusual feature of DEC-205, which in turn leads to clearly improved antigen processing and MHC class II presentation to $CD4^+$ T cells compared to antigen uptake by the MMR, which lacks the EDE region. Although DEC-205 contains ten CRDs as well as a cysteine-rich domain, the receptor is not able to bind sugars as shown for other members of the C-type lectin family (Mahnke et al. 2000; East & Isacke 2002).

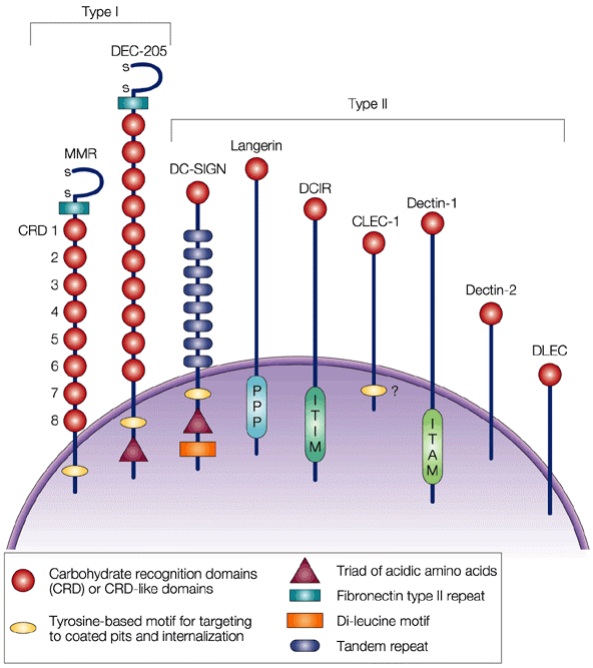

Figure 8. DEC-205 and the C-type lectin receptors expressed by dendritic cells.

C-type lectin receptors expressed by DCs are summarized. See text for detailed description. Abbreviations: C-type lectin receptor-1 (CLEC-1); cysteine-rich domain (CR); dendritic cell (DC); dendritic cell immunoreceptor (DCIR); DC-specific ICAM-3 grabbing non-integrin (DC-SIGN); DC lectin (DLEC); immunoreceptor tyrosine-based activation motif (ITAM); immunoreceptor tyrosine-based inhibitory motif (ITIM); macrophage mannose receptor (MMR); proline-rich regions (P). *Source: Figure was taken from Figdor et al. 2002.*

In mice, DEC-205 is expressed at high levels on $CD11c^{+}CD8^{+}$ lymphoid-tissue resident cDCs present in the thymus, spleen and lymph nodes as well as on dermal, interstitial Langerhans migratory cDCs (Fig. 5) (Vremec & Shortman 1997; Vremec et al. 2000; Heath et al. 2004; Shrimpton et al. 2009). Moreover, it is also present at very low levels on B cells, T cells and granulocytes. Whereas the expression profile is relatively DC restricted in mice, it is less restricted in humans (Inaba et al. 1995; Kato et al. 2006; Tacken et al. 2007). Human DEC-205 is highly expressed on myeloid DCs and monocytes. But in contrast to mice, it is also, to a smaller extent, expressed by several other immune cells such as B cells and even less by NK cells, pDCs and T cells. Nevertheless, the structure and function of DEC-205 seems to be conserved between the species, since the human DEC-205 protein exhibits ~80 % identity to its mouse homolog. Even though the exact physiological ligands of DEC-205 are not known to date, a lot of studies using DEC-205-specific antibodies as surrogate ligands have been performed mostly in murine systems, where DEC-205 expression is almost exclusively restricted to DCs (Kato et al. 1998; McKay et al. 1998; East & Isacke 2002, Kato et al. 2006). Whereas mAbs such as the mouse MR6, MG38 and MMRI-7 have been used to increase our knowledge regarding the specific function of the human DEC-205 molecule, the rat-derived mAb NLDC-145 was not only the first antibody found to bind mouse

DEC-205, but has also been utilized extensively to investigate the function and specialized properties of DEC-205 in general (Kraal et al. 1986; McKay et al. 1998; Guo et al. 2000; Kato et al. 2006). Several publications impressively demonstrated that NLDC-145, when genetically or chemically conjugated to an antigen, successfully targets DCs *in vivo*, leading to highly efficient endocytosis of the antigen and its subsequent processing and presentation on both MHC I and MHC II molecules. The fact, that DEC-205-mediated antigen targeting to DCs results in the induction of more efficient $CD4^+$ and $CD8^+$ T cell responses than other DC targeting strategies, is considered to be a consequence of the specialized function of DEC-205 to deliver antigen to the late endosomes and to allow cross-presentation (Mahnke et al. 2000; Bonifaz et al. 2002; Bonifaz et al. 2004; Trumpfheller et al. 2006; Bozzacco et al. 2010). Moreover, the outcome of DC targeting and therefore the kind of T cell response induced can easily be biased depending on the presence or absence of an additional DC activation signal. Hawiger et al. and Bonifaz et al. demonstrated that antigen delivery to $DEC\text{-}205^+$ immature DCs without the addition of an inflammatory stimulus induces tolerance to the antigen (Hawiger et al. 2001; Bonifaz et al. 2002). Following T cell stimulation by immature DCs, tolerance is mediated due to the induction of T cell anergy and more importantly the induction of regulatory T cells, which confer active immunosuppressive functions (Mahnke et al. 2003). In addition, there is experimental evidence that a specific subset of splenic DCs ($CD8^+$), which also expresses DEC-205, is involved in mediating tolerance (Kronin et al. 2000). Together, these data prompted researchers to open the field for using DEC-205 as an attractive target for tolerance induction to auto-antigens and allergens (Petzold et al. 2010). Bruder et al. successfully implemented this idea and demonstrated that DEC-205 targeting prevents the onset of type I diabetes in a mouse model (Bruder et al. 2005). In direct contrast to the observed tolerogenic effect of DEC-205-mediated targeting of antigens to immature DCs, long-lived immunity mediated by highly efficient and antigen specific $CD4^+$ and $CD8^+$ T effector cells is induced when DC maturation stimuli are co-administered together with the antibody targeting the antigen to DEC-205 (Fig. 9) (Bonifaz et al. 2002; Bonifaz et al. 2004; Hawiger et al. 2001).

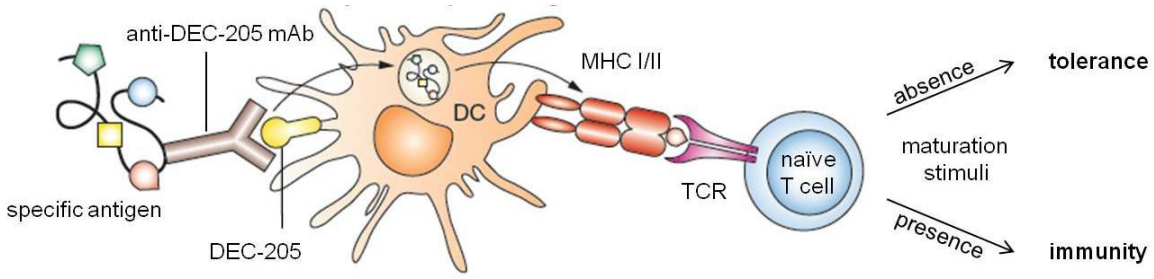

Figure 9. DEC-205 targeting in dendritic cell-based immunotherapy.

DEC-205 on DCs can be targeted *in vivo* using a specifically binding monoclonal antibody (mAb) such as NLDC-145, which has been conjugated to an antigen. Tolerance or immunity can be induced depending on the absence or presence of maturation stimuli. See text for detailed description. Abbreviations: major histocompatibility complex I/II (MHC I/II); T cell receptor (TCR). *Source: Figure was modified from Tarner & Fathman 2006.*

The observation that DEC-205 targeting leads to robust immunity in the presence of DC maturation stimuli was the basis for developing successful vaccination protocols against viral and cancer antigens in mice (Mahnke et al. 2005; Trumpfheller et al. 2006; Bozzacco et al. 2007; Gurer et al. 2008; Johnson et al. 2008) and DEC-205 has become a prime target for various immunotherapeutic approaches. In the course of these studies a lot of different DEC-205/antigen constructs have been developed, all of which take advantage of the exceptional capacity of DEC-205 targeting of antigen to DCs (Tab. 3). Initial constructs were based on chemical conjugation of a given antigen to the DEC-205-specific antibody and were successfully used to target the model antigen OVA as well as melanoma antigens (TRP-2, gp100) or Ag85B from *mycobacterium tuberculosis* to DCs *in vivo*. Newer genetic approaches are based on the generation of recombinant fusion proteins resulting from insertion of the specific antigen into the carboxyl terminus of the heavy chain of the anti-DEC-205 mAb (αDEC-205) (Tab. 3). On the basis of this approach, Bozzacco et al. were the first ones to show that *in vitro* loading of human DCs with αDEC-205/HIVgag p24 fusion protein results in cross-presentation of several different MHC I peptide epitopes derived from a single protein (Bozzacco et al. 2007). Moreover, targeting DEC-205 on human DCs with the αDEC-205/EBNA1 fusion protein (Epstein-Barr virus nuclear antigen 1) expanded pre-existing protective EBNA1-specific $CD4^+$ and $CD8^+$ memory T cells against Epstein-Barr virus (EBV) in mice reconstituted with components of the human immune system (Gurer et al. 2008). In addition to these fusion proteins comprising the antigen and the entire antibody molecule, Johnson et al. generated a DEC-205-specific single-chain Fragment variable (scFv) that lacks the constant region of the antibody but contains the variable heavy (V_H) and light (V_L) chain fused to gp100, the melanoma tumor-associated antigen (Johnson et al. 2008). Immunization with the αDEC-205/gp100-scFv decreases the growth rate of an aggressive form of melanoma, thereby underlining the therapeutic potential of antigen

delivery to DCs via αDEC-205/antigen-scFv. Other groups took advantage of vectors encoding for HIVgag p41 fused to a DEC-205-specific scFv. Injection of these DNA vaccines in combination with appropriate adjuvants led to improved $CD8^+$ T cell responses when compared to control vectors (Tab. 3) (Nchinda et al. 2008; Grossmann et al. 2009; Nchinda et al. 2010; Maamary et al. 2011). In conclusion, DEC-205 represents a very attractive target for therapeutic vaccination against cancers and pathogens establishing chronic infections.

Construct	Species	Antigen		Reference
mAb chemically conjugated to antigen	mouse	OVA		Bonifaz et al. 2002 Mahnke et al. 2003 Bonifaz et al. 2004
		TRP-2 gp100	melanoma	Mahnke et al. 2005
		Ag85B	*mycobacterium tuberculosis*	Stylianou et al. 2011
mAb fused to antigen	mouse	OVA		Boscardin et al. 2006 Flacher et al. 2010
		HIVgag p24 HIVgag p41	HIV	Trumpfheller et al. 2006 Bozzacco et al. 2007 Trumpfheller et al. 2008 Bozzacco et al. 2010 Idoyaga et al. 2011
		LcrV-Protein	*Yersinia pestis*	Do et al. 2010
	rhesus macaques	CSP	*Plasmodium falciparum*	Tewari et al. 2010
	human	HIVgag p24	HIV	Bozzacco et al. 2007
		EBNA1	Epstein-Barr virus	Gurer et al. 2008
		NY-ESO-1	cancer testis	Tsuji et al. 2011
scFv fused to antigen	mouse	gp100	melanoma	Johnson et al. 2008
	human	MAGE-A3	cancer testis	Birkholz et al. 2010
DNA vaccine (DEC-205 scFv + virus vector)	mouse	OVA HIVgag p41	HIV	Nchinda et al. 2008 Grossmann et al. 2009 Nchinda et al. 2010 Maamary et al. 2011

Table 3. Overview of αDEC-205/antigen constructs.

Selected so far used αDEC-205/antigen constructs are summarized. Abbreviations: circumsporozoite protein (CSP); Epstein-Barr virus nuclear antigen 1 (EBNA1); monoclonal antibody (mAb); ovalbumin (OVA); single-chain Fragment variable (scFv); tyrosinase-related protein (TRP-2).

2.4 Hepatitis C virus

Among the many viruses that are known to infect the liver, HCV is one of the most important ones because of its aggressive capacity to cause persistent liver infection leading to cirrhosis and hepatocellular carcinoma (HCC) in the further course of the disease. Thus, HCV displays a serious worldwide public healthcare problem, the importance of which is likely to even increase over the coming years. There are 3 to 4 million new cases of HCV infection each year and current estimates suggest that approximately 170 million individuals are chronically infected (WHO). Moreover, HCV infection is the leading indication for liver transplantation in the United States and Europe and it is five times as widespread as infection with HIV (Lauer et al. 2001; Brown et al. 2005). Since the discovery of HCV in 1989 (2.4.1), detailed information regarding the viral particle, its molecular organization and replication, epidemiology and pathogenicity have become available. Despite this thorough knowledge, until now no effective vaccine exists, thus indicating the urgent need for developing an efficient immunotherapy against HCV infection (Leroux-Roels 2005).

2.4.1 History

To date, there are five human hepatitis viruses identified, A, B, C, D and E, of which A, B and C are the most common types (Woltman et al. 2010). After development of serological tests to detect hepatitis A virus (HAV) or hepatitis B virus (HBV) infections in patients in the 1970s, it became clear that many cases of post-transfusion hepatitis could not be explained by these two particular infectious agents. This led to the designation of an at this time still unidentified hepatitis virus, which caused “non-A, non-B” hepatitis and which was shown to be readily transmittable to chimpanzees. In 1989, the great breakthrough was made as HCV genomic clones could be isolated and identified as the cause for “non-A, non-B” hepatitis (Choo et al. 1989). Since it was the third discovered virus of the *Hepacivirus* genus in the *Flaviviridae* family, it was named HCV (Robertson et al. 1998).

2.4.2 The hepatitis C virus genome and its genetic diversity

HCV is a small enveloped RNA virus possessing a single-stranded, positive strand genome (ss(+)RNA) and belonging to the *Hepacivirus* genus of the *Flaviviridae* family. The 9.6 kb genome of HCV consists of a single open reading frame that codes for a polyprotein of approximately 3010 amino acids (aa) and is flanked by a highly conserved non-coding region (NCR) at the 5´ and 3´ ends, respectively. The 5´NTR contains thereby an internal ribosome entry site (IRES), which mediates the polyprotein expression. The polyprotein precursor collectively encodes for ten structural and non-structural proteins, which are released as mature proteins after translation and processing by host and viral proteases (Bartenschlager et al. 2004; Dustin & Rice 2007; Moradpour et al. 2007). The structural proteins include the

capsid protein Core and two envelope glycoproteins (E1 and E2) that mediate binding to co-receptors and entry into hepatocytes (Pileri et al. 1998; Scarselli et al. 2002; Evans et al. 2007). Moreover, the E2 includes two regions with extremely high sequence variability, the hypervariable regions 1 and 2 (HVR 1 and HVR 2), which are thought to be the result of selection pressure conferred by virus-specific antibodies (Kato et al. 1992; Lauer et al. 2001). The nonstructural proteins possess various functional properties such as ion channel (p7), protease (NS2, NS3, NS4A), RNA helicase (NS3) and RNA-dependent RNA polymerase (NS5B) activity. The roles of NS4B and NS5A are not yet fully understood in detail. In general, the nonstructural proteins are involved in viral replication and particle formation (Fig. 10) (Dustin & Rice 2007; Moradpour et al. 2007).

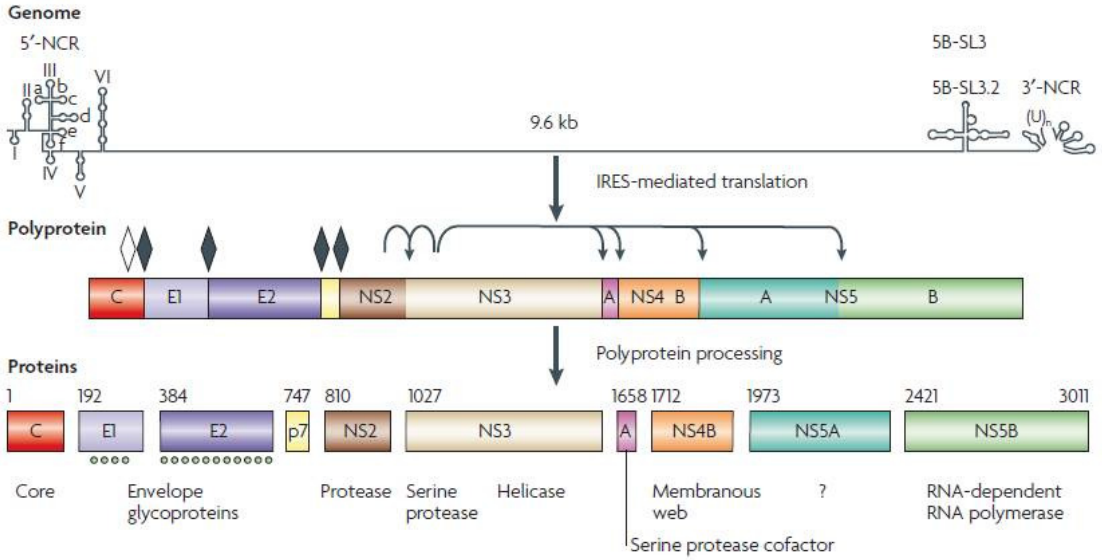

Figure 10. The hepatitis C virus genome and the processing into structural and nonstructural proteins.

The organization of the HCV genome and the processing of the polyprotein into the ten structural (Core, E1, E2) and non-structural (p7 - NS5B) proteins are shown. See text for detailed description. Abbreviations: hepatitis C virus (HCV); internal ribosome entry site (IRES); non-coding region (NCR); ribonucleic acid (RNA). *Source: Figure was taken from Moradpour et al. 2007.*

Like other viruses such as HIV, HCV is characterized by its high genetic variability. A combination of two reasons is considered to account for this effect: on the one hand, the viral replication is extremely robust and it is estimated that more than 10^{12} virions are produced per day in a given individual (Neumann et al. 1998). On the other hand, the replicative machinery is error prone, since replication occurs through an RNA-dependent RNA polymerase that lacks a “proofreading” function. This results in a heterogeneous mixture of a high number of divergent RNA genomes that coexist in an infected individual, the so called quasispecies. Both the high turnover rates and the existence of quasispecies may explain the rapid emergence of viral diversity in infected persons and the frequent persistence of

infection as a result of immune escape (Kew et al. 2004). Besides the genetic variation within an individual patient, 6 major genotypes (genotype 1-6) that differ in their nucleotide sequence by 30-35 % can be classified in addition to several subtypes (a, b, c etc.), which in turn show 75-80 % sequence overlap (Simmonds et al. 1993; Simmonds et al. 2005). These genotypes are variably distributed over the world, with genotypes 1a and 1b occurring most commonly in the United States and Western Europe, whereas genotype 6 is virtually never found in these countries, but is common in Southeast Asia. Knowledge regarding the distribution of HCV genotypes plays an important role since they all have the potential to cause severe liver disease and because the genotype largely determines the duration and dosage of antiviral therapy and influences its outcome (Wasley & Alter 2000; Lauer et al. 2001).

2.4.2.1 Structure and function of selected hepatitis C virus proteins: Core and NS3

- ***Core***

The Core protein is located at the N-terminus of the HCV polyprotein and forms the viral nucleocapsid. In comparison to other HCV proteins the aa sequence of Core is highly conserved among different HCV genotypes, which implies an important biological function. Cleavage of the signal sequence by the signal peptidase yields an immature 191 aa Core protein, which leads to the mature 21 kDa Core protein by further C-terminal processing (Hijikata et al. 1991; Yasui et al. 1998). While the C-terminal part of the Core protein is primarily hydrophobic, the N-terminal part is highly hydrophilic due to a high proportion of basic aa residues (McLauchlan et al. 2000). The location of the Core protein is restricted to the cytoplasm, where it is associated with the ER membrane or located at the surface of lipid droplets, which may be required for correct viral particle morphogenesis. Furthermore, the Core protein has been shown to act in a multifunctional way, since it is involved in the modulation of gene transcription, cell proliferation, cell death and in the pathogenesis of the viral infection by suppressing host immune responses (Penin et al. 2004; Boulant et al. 2006; Miyanari et al. 2007; Moradpour et al. 2007). Core is among the most conserved genes in various HCV genotypes with several well-characterized B cell, T cell, and CTL antigenic determinants. Moreover, the presence of Core-specific CTLs is shown to enhance the beneficial effect of IFN therapy in infected patients. Therefore, the HCV Core protein is an attractive candidate for inclusion in vaccine design for both therapy and prophylaxis against HCV infection (Christie et al. 1999; Lechner et al. 2000).

- ***NS3***

The full-length NS3 protein spans aa 1027 to 1658 on the HCV polyprotein (genotype 1b) and comprises dual activities: it contains a serine-protease (189 aa; 20.8 kDa) (aa 1027-1216) at the N-terminal end of the protein and an RNA helicase/NTPase (442 aa) (aa 1217-1658; 48.6 kDa) at the C-terminus (Penin et al. 2004; Raney et al. 2010). The N-terminal domain of NS3 displays an integral part of the NS2-NS3 proteinase, which undergoes autocatalytic cleavage to produce NS2 and NS3. Subsequently, the remaining NS3 is released by the NS3 protease and forms the NS3-4A serine proteasecomplex consisting of a catalytic subunit (NS3 protein) and an activating cofactor, the NS4A protein. The NS3-4A serine protease is responsible for further processing of the HCV polyprotein. In contrast, the C-terminal domain of the NS3 protein includes an RNA helicase and NTPase that is unrelated to the serine protease and the HCV helicase activity seems to be necessary for viral replication (Yao et al. 1999; Penin et al. 2004; Frick 2006). As for the Core protein, the sequence of the NS3 protein is also more conserved between genotypes than other HCV proteins (Leroux-Roels 2005). Due to the relatively low sequence variability and the multifaceted function of the NS3 protein being essential for replication and translation of viral RNA and polyprotein processing, this HCV protein is considered to be an attractive target in the design of anti-HCV vaccine (Frelin et al. 2003; Isaguliants et al. 2003). In addition, since the NS3 proteins contains an immunodominant $CD4^+$ T helper epitope and several CTL epitopes, which have been associated with control of HCV in patients with self-limiting infection, it represents a promising vaccine candidate expected to induce both $CD4^+$ and $CD8^+$ T cell-mediated protective immune responses (Battegay et al. 1995; Kurokohchi et al. 1996; Diepolder et al. 1997).

2.4.3 Infection routes

HCV is a blood-borne virus; therefore, the most important source of HCV infection in developed countries during the 1970s and 1980s was a consequence of parenteral blood transfusion or the use of injectable drugs. Since the very early 1990s HCV transmission by blood was significantly reduced especially through improved blood-screening measures based on the search for general blood-borne viruses, the detection of HCV antibodies as well as the use of surrogate markers in donor blood samples. Moreover, the use of virus inactivation steps in the manufacturing process of blood products has nearly eliminated transmission of HCV by clotting factor concentrate and other blood products. Additionally, needle exchange programs and education among injecting drug users has been performed to reduce the number of new cases of HCV infection. Today, these efforts and the introduction of routine testing of donated blood have not only dramatically decreased the risk of transfusion-associated HCV infection, but also virtually eliminated transmission of HCV by

this route in the developed countries. Nevertheless, the predominant risk factor in developed countries is the blood-to-blood contact in conjunction with intravenous drug use combined with contaminated needles or sharps. Sexual and vertical (mother-to-child) transmission plays a minor role, whereby especially the latter shows only a very low risk. In case of sexual contact, transmission is limited and only occurs in high-risk settings such as concomitant HIV infections. In contrast, the major source of HCV infection in the developing countries are unsafe injections and ineffective injection control practices, so that nosocomial transmission displays a not uncommon route and may be vastly underestimated. Other ways of transmission include the use of unsterilized objects for rituals (e.g. circumcision, scarification), traditional medicine (e.g. blood-letting), other activities that break the skin (tattooing, body piercing) as well as intravenous drug abuse (Donahue et al. 1992; NIH 1997; WHO 1999; Kew et al. 2004; Maheshwari et al. 2008). In conclusion, despite the mentioned activities to prevent HCV infection, the number of newly infected individuals remains at an alarmingly high level with 3 to 4 million new cases each year. Therefore, the necessity for an effective vaccine is evident.

2.4.4 Clinical characteristics and natural course of the disease

The course of acute HCV infection is generally associated with either no symptoms or mild symptoms indicated by jaundice, malaise, nausea, fatigue and low-grade fewer. Since clinical manifestations show only mild symptoms within 7 to 8 weeks after HCV exposure, the majority of patients do not realize the infection and progress to chronic viremia in 70-90 % of the cases. Therefore, acute infection is only infrequently diagnosed, which is a crucially problematic factor in the treatment of the infection as well as in the development of effective vaccines. However, 10-30 % of acutely infected patients achieve spontaneous viral clearance and studies suggest that this elimination of the virus is associated with strong symptoms during the acute phase of the infection (NIH 1997; Lauer et al. 2001; Maheshwari et al. 2008). For HCV elimination, humoral immune responses seem to have little effect on the clearance of viremia, whereas several studies suggested that vigorous $CD4^+$ and $CD8^+$ T cell-mediated immunity plays an important role (Lechner et al. 2000; Thimme et al. 2001; Bartosch, Bukh et al. 2003; Bowen et al. 2005). In the case of insufficient initial immune responses, the acute HCV infection becomes persistent and turns to a chronic manifestation. This phase is typically characterized by a prolonged period without or with only mild symptoms. Most notably, the immune escape mechanisms of the virus based on its high replication rate and the occurrence of quasispecies are thought to contribute to the attenuated, clinically asymptomatic course of the acute and chronic HCV infection (Takahashi M. et al. 1993; NIH 1997; Rehermann & Nascimbeni 2005). Therefore, a balance between weak immune responses and virus escape predominates during this phase so that

most of the chronically infected individuals show a slow progression of the disease with tendency to hepatitis and to some degree of fibrosis, if any. However, 20 % of chronically infected patients develop severe complications like liver cirrhosis followed by hepatocellular carcinoma (HCC), liver failure and death, usually detectable in the second or third decade of the infection (NIH 1997; WHO 1999; Lauer et al. 2001). Since HCV shows no cytopathogenic activity, hepatocyte damage during viral hepatitis seems to be mediated by the host's cellular immune response to the infection (Guidotti & Chisari 2006). In addition, factors that influence the rate of progression of chronic hepatitis C to cirrhosis and HCC include alcohol abuse, age at the time of infection, severity of liver histology at initial biopsy and viral titer (Fig. 11) (WHO 1999).

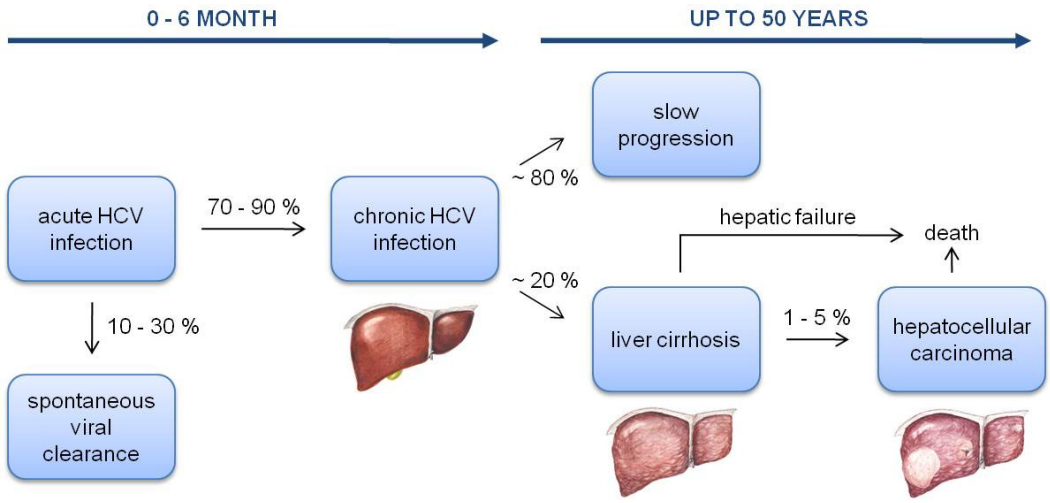

Figure 11. Hepatitis C virus infection and disease pathogenesis.

Infection with the hepatitis C virus (HCV) will result in different outcomes displayed in the figure. See text for detailed description. *Source: Figure was adapted from Woltman et al. 2010.*

2.4.5 Treatment

The propensity of HCV to cause chronic infection unlike no other hepatitis virus makes the virus especially difficult to treat (Lang & Weiner 2008). However, some standard treatments have been developed, which are not able to induce viral clearance, but slow down the progress of the disease and significantly improve antiviral responses. Current standard therapy of HCV-infected patients includes the IFNα monotherapy. IFNα is a cytokine with important functions in the innate immune response towards viruses. Once infected, immune and other cells produce and secrete IFNα, which is able to bind to IFN sensitive receptors on adjacent cells. This activation sets off a signaling cascade which results in inhibition of viral replication, upregulation of MHC-II on host cells and activation of NK cells (Davis et al. 1989; McHutchison et al. 1998; Manns et al. 2007; Lang & Weiner 2008). As a consequence of the fact that the treatment effectivity is 50-70 % during the acute phase, while it declines to about

30 % in patients with minimal or mild chronic hepatitis and to about 10 % among those with cirrhosis, the IFNα therapy should commence as early as possible after the initial infection (WHO 1999). Although the detailed mechanisms of IFNα therapy are still incompletely understood, it is thought that the cytokine exerts a direct antiviral effect by boosting the host immune responses against the virus. The combination of IFNα and ribavirin, a nucleoside analog with broad activity against viral pathogens, seems to intensify the effects of IFNα. Moreover, ribavirin has been proven beneficial, since it prevents relapse after the end of antiviral treatment. Nevertheless, the half-life of IFNα is a limiting factor in the therapy that has been tried to improve. One opportunity is the pegylated interferon alpha (PEG-IFNα), which is already in use combined with ribavirin to treat HCV infected patients. The advantage of PEG-IFNα compared to unpegylated IFNα is that it shows not only prolonged half-life to approximately 40-80 hours, but also increased sustained viral responses (Manns et al. 2001; Fried et al. 2002; Manns et al. 2007). Recently, two other therapy forms have been evaluated in clinical studies, these are albumin-conjugated IFNα (albIFNα) (Balan et al. 2006) and consensus IFN (CIFN) (Sjogren et al. 2005). Both seem to be promising candidates for treatment of chronic HCV, since they are well tolerated and extend sustained viral responses, even in patients infected with HCV genotype I, which has shown insufficient response to the previous standard treatments (Lang & Weiner 2008). Despite the successes with the standard and new treatment protocols, morbidity and mortality rates associated with HCV are predicted to rise in the coming years. In general, current treatment options are limited by several factors. Not only the viral genotypes play an important role, since they differently respond to certain therapy forms, but also patient characteristics influence therapy outcomes, in particular if they have failed to respond to previous treatment or suffer from severe liver fibrosis and cirrhosis. Further important facts are the cost intensity of current treatment options and the induced side effects including influenza-like symptoms, anemia and depression. Again, this clearly illustrates the urgent need for a development of vaccines that help achieve the ultimate goal of HCV therapy – the complete elimination of the virus in all patients (Manns et al. 2007).

2.4.6 Model systems

HCV studies are associated with practical obstacles that have enormously hindered investigations of the virus itself as well as the development of vaccines due to the lack of suitable tissue culture and animal model systems. First studies concerning the viral entry and antibody-mediated neutralization could not be performed until 2003, when retroviral particles pseudotyped with HCV proteins were developed (Bartosch, Dubuisson et al. 2003; Bartenschlager et al. 2004). The establishment of a tissue culture model based on recombinant cell culture derived HCV infecting a human hepatoma cell line (huh-7) has not only been a breakthrough for HCV research, but has also allowed the characterization of the entire viral life cycle and viral-host interactions in detail (Heller et al. 2005). Nevertheless, studies on HCV pathogenesis have been impeded due to the lack of suitable animal model systems. Based on the fact that wild-type mice cannot be infected with HCV, only transgenic mice expressing HCV genes in the liver or partially humanized immunodeficient mice with e.g. chimeric human livers are in use. Except humans, chimpanzees are the only natural animal model susceptible for infection with HCV. Studies in chimpanzees have generated extremely valuable knowledge of HCV characteristics such as viral transmission, replication and immune responses during the infection and have been proven very useful in the preclinical phases of vaccine development. Although these animals are a valuable model system providing important information on HCV biology, animal studies in primates are associated with ethical issues, limitations in numbers of animals and are highly cost-intensive to maintain (Dustin & Rice 2007; Halliday et al. 2011).

2.4.7 Immune responses to the hepatitis C virus

The interaction between HCV and its host is a dynamic process, in which on the one hand the virus pursues to decrease its visibility and on the other hand the host attempts to prevent and eliminate infection while minimizing collateral damage to itself (Guidotti & Chisari 2007). At some point during the infection the host immune system recognizes the virus and elicits humoral and cellular immune responses, which are of varying intensities and complexities. As described earlier, only 10-30 % of infected individuals undergo spontaneous recovery in the acute phase of infection, whereas in 80 % of the cases the infection develops into a chronic phase (2.4.4). This effect has been accounted to the variable intensity of the mounted immune responses (Leroux-Roels 2005). Several studies demonstrate that a strong, multi-specific (against several viral epitopes) and sustained HCV-specific T cell response is correlated with viral clearance during acute HCV infection (Missale et al. 1996; Chang et al. 2001) and during which $CD8^+$ T cells are the major effector cells mediating protective immunity. However, also $CD4^+$ T cells seem to play a role, since it has been shown that $CD8^+$ T cells alone will not achieve protection from this quickly mutating virus

(Grakoui et al. 2003). Moreover, activated T cells secrete proinflammatory cytokines such as IFNγ, which directly enhance the intracellular inhibition of viral replication (Frese et al. 2002). In contrast, in patients developing chronic hepatitis, the corresponding immune response is weak and inadequate, but ongoing, which is considered to be the cause of the progressive liver cell damage and chronic hepatic inflammation that ultimately leads to fibrosis and cirrhosis (Fig. 11). Various mechanisms have been suggested to underlay the diversity of the outcome of HCV infection, which are mainly T cell exhaustion, viral escape from T cell recognition and the tolerogenic environment of the liver (Halliday et al. 2011). The mechanisms underlying exhaustion are poorly understood, but the inhibitory receptor programmed-death 1 (PD-1) on T cells seems to be involved (Barber et al. 2006). This suggestion was confirmed by results from Radziewicz et al., which clearly showed that PD-1 is upregulated on the majority of T cells from the liver, the site of viral replication, of chronic HCV infected individuals in comparison with those circulating in the peripheral blood (Radziewicz et al. 2007). Recently, it could further be demonstrated that a complex interplay of immunological and virological factors such as the state of T cell differentiation and ongoing antigen triggering determine the observed T cell exhaustion in human chronic HCV infections (Bengsch et al. 2010). Besides this, mechanisms influenced by the virus itself have been considered to play an important role. HCV comprises the capability to escape from the host immune system through genetic variation (2.4.4) and in addition has developed various strategies to interfere with the host by impairing both innate and adaptive immune responses. One mechanism of interaction is that the virus undermines the potent antiviral effects of IFNs (Leroux-Roels 2005). Thereby, HCV proteins interfere with signaling pathways in a manner to inhibit expression of IFN genes. For example the NS3/4A serine protease blocks the phosphorylation of IFN regulatory factor (IRF)-3, the key molecule in antiviral IFN signaling (Foy et al. 2003). On a cellular level, NK and NKT cells are important in the early innate immune responses against viral infection, since they recognize infected cells and perform cytotoxic lysis by releasing perforin and proteases. It has been shown that HCV influences the activity of NK cells by blocking their activation, cytokine secretion and cytotoxic activity mediated though the binding to their surface (Ahmad & Alvarez 2004; Leroux-Roels 2005; Cãruntu & Benea 2006). In addition, also adaptive immune responses are impaired by the virus. HCV readily produces escape mutants to CTLs and inhibits the binding of virion-neutralizing antibodies though lipoprotein masking (Erickson et al. 2001; Lavilette et al. 2005). Finally, as mentioned above, the liver itself could also play an important role, since it is believed that a tolerogenic environment predominates in this organ, primarily induced by liver sinusoidal endothelial cells (LSECs) (Dustin & Rice 2007; Halliday et al. 2011). Therefore, if T cell priming occurs within the liver, the priming is more likely to induce T cell inactivation, tolerance or apoptosis (Bertolino et al. 2002; Crispe 2003). In an evolutionary

view, this could be explained by the needed protection of the liver, which is constantly exposed to antigens via the portal tract. The mentioned PD-1 upregulation on T cells from the liver, which indicates an exhausted phenotype, could be caused by the special conditions in this organ. This would in turn mean that antiviral T cells primed in the periphery during vaccination could be of a “superior” quality to those primed in the liver, which seem to be impaired in their effector functions against appropriate targets (Dustin & Rice 2007; Halliday et al. 2011).

In conclusion, the mechanisms behind spontaneous viral clearance in contrast to persistence remain only partially understood. However, there is strong evidence that the outcome of HCV infection is modulated by a complex interplay of different factors regarding the liver, the immune system and the virus, such as a tolerogenic environment, impaired innate and adaptive immune responses, T cell exhaustion, viral escape mutations or immune inhibitory viral proteins (Post et al. 2009).

2.4.8 Basis for the study

The development of effective vaccines against HCV has long been defined as a difficult venture and was considered only an unlikely possibility still a decade ago. The major scientific challenges result from the genetic diversity of the virus, from evidences that convalescent humans and chimpanzees could be readily re-infected, from the lack of reliable small animal systems and from the high grade of chronic persistent HCV infections. However, recent studies and observations suggest hope for the development of prophylactic and therapeutic vaccines, at least for the generation of a partly effective vaccine avoiding chronic progress of the disease. First, the general possibility of spontaneous viral clearance in a portion of infected patients, which is associated with vigorous and sustained specific immune responses to the virus, provides a realistic option for therapeutic exploitation. Second, natural immunity against the virus seems to exist, since it has been shown that the risk of developing chronic disease following HCV re-infection is markedly reduced compared to that following primary infection even with very divergent viral strains, which is correlated with both $CD4^+$ and $CD8^+$ T cell responses (Berzofsky et al. 2004; Leroux-Roels 2005; Houghton & Abrignani 2005; Stoll-Keller et al. 2009).

So far, several HCV vaccination strategies were successfully tested in animals including vaccines based on recombinant proteins, peptides, viral vectors and DNA (DNA vaccine). Some of those strategies have already been tested in clinical studies or are underway. Moreover, future vaccines such as virus-like particle (VLP)-based vaccines that have been proven to be successful for inducing immunity to HBV will be promising alternatives (Halliday et al. 2011). However, despite these enormous efforts to design an effective vaccine, no such product is available for humans to treat the HCV infected patient; thus the search

proceeds. Based on the current knowledge of the mechanisms involved in control of viral infection, immunization with an effective HCV vaccine should induce a broad range of cross-neutralizing antibodies to inhibit viral spread combined with multi-specific and vigorous cellular immune responses, including both $CD4^+$ and $CD8^+$ T cells, to clear the virus and to destroy virus infected hepatocytes (Stoll-Keller et al. 2009). Given the central role of DCs in orchestrating innate and adaptive immune responses, they may represent an exceptionally attractive target for the development of an effective HCV vaccine (Fig. 12).

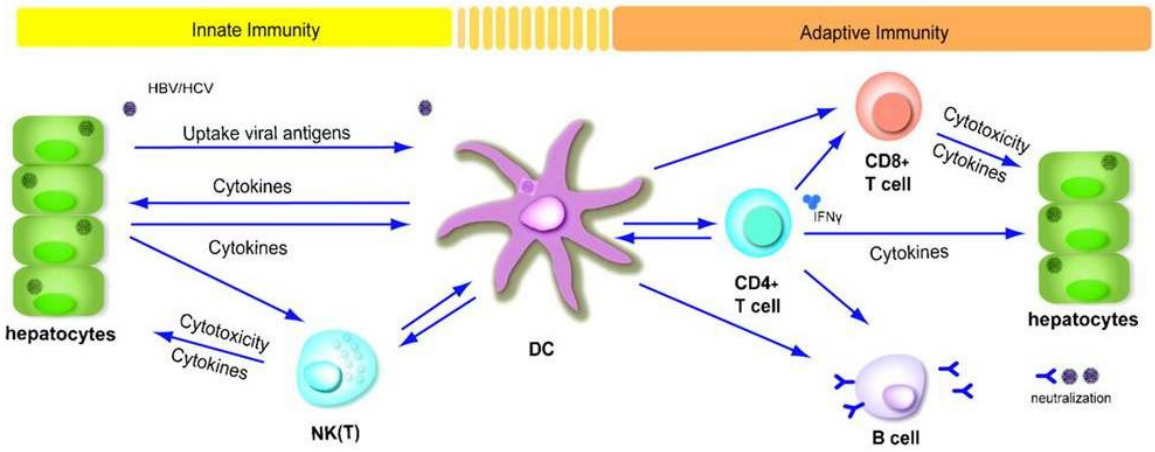

Figure 12. Dendritic cells as targets for therapeutic vaccination against hepatitis C virus

DCs as the central players in the regulation of antiviral immunity provide not only a bridge between innate and adaptive immune responses, but also combine all elements of the immune system needed for designing an effective vaccine against HCV. The most professional APCs are able to take up viral antigens from infected hepatocytes. Subsequently, various cellular and humoral immune responses are initiated: NK and NKT cells and specific cytokines within innate immunity and $CD4^+$, $CD8^+$ T cells as well as B cells on the adaptive side of immunity. Abbreviations: antigen-presenting cells (APC); dendritic cell (DC); hepatitis C virus (HCV); natural killer cells (NK cells); natural killer T cells (NKT cells). *Source: Figure was taken from Woltman et al. 2010.*

3 Aims of the study

The pivotal role of DCs in mediating immunity to pathogenic microorganisms and the recent advances in exploiting their functions for applications in the clinic have made them an exceptionally attractive target for vaccination. Here, DCs may provide an opportunity to treat infectious diseases against which effective vaccines and therapies are still lacking, such as HCV. The overall aim of this thesis was to test different *in vivo* DC targeting strategies with respect to their applicability for inducing antiviral immunity in the liver and thus to provide the basis for the development of a DC-based HCV vaccine. As a prerequisite for this goal, first the differences between two distinct *in vivo* DC targeting strategies, utilizing either the endocytosis receptor DEC-205 or the TLR2/6 heterodimer, were to be investigated using a model antigen. In the second part of the presented work, a DC-based vaccination strategy against HCV infection was to be designed.

To gain detailed knowledge of the triggered immune responses which would later be useful for effective HCV vaccination αDEC-205/antigen conjugates were to be compared to BPPcysMPEG, the synthetic derivative of MALP-2, which was either separately delivered with the entire protein antigen or directly linked to the immunodominant MHC-I and MHC-II peptide epitopes (BPPcysOVAMPEG). For this, effective generation of αDEC-205/OVA conjugates, consisting of a DEC-205-specific antibody and the model antigen OVA, needed to be established first, especially with regard to the conjugation of HCV proteins later in the study. Afterwards, immunization studies were to be carried out to analyze the influence of both DC targeting strategies on the stimulation of antigen-specific cellular and humoral immune responses. To address the question whether targeting DEC-205 or the TLR2/6 heterodimer would also be effective to induce antiviral immunity in the liver, additional studies in a model using OVA-expressing recombinant adenovirus as a surrogate for HCV liver infection were to be performed. The results of these investigations with model antigens were analyzed to define optimal conditions for an immunotherapy against HCV infection and thus were to provide the basis for the second part of the project. Therefore, the detailed analyses of DEC-205 targeting in the context of anti-viral therapy were to be extended to design an effective DC-based HCV vaccine. To this end, the selected HCV proteins NS3 and Core were to be purified and conjugated to αDEC-205. The resulting conjugates were then to be characterized regarding their ability to bind DEC-205 on DCs. Additionally, first immunization trials with both αDEC-205/NS3 and αDEC-205/Core were to be performed in mouse studies in order to assess the induced HCV-specific immune responses.

4 Materials and Methods

4.1 Materials

4.1.1 Antibodies

Specificity	Isotype	Conjugation	Clone	Source
ELISA				
α-mouse IgG	goat	HRPO	-	Dianova (Germany)
α-mouse IgG	goat	Biotin	polyclonal	Sigma-Aldrich (Germany)
α-rat IgG	donkey	HRPO	-	Jackson ImmunoResearch (United Kingdom)
HCV Core	mouse IgG_1	purified	C7-50	Thermo Fisher Scientific (Germany)
HCV NS3	mouse IgG_1	purified	20-8	Thermo Fisher Scientific (Germany)
Streptavidin	-	HRPO	-	BD Biosciences (Germany)
ELISPOT				
α-mouse IFNγ	rat IgG_1	Biotin	R4-6A2	eBioscience (Germany)
α-mouse IFNγ	rat IgG_1	purified	AN-18	eBioscience (Germany)
α-mouse IL-4	rat IgG_1	Biotin	-	BD Biosciences (Germany)
α-mouse IL-4	rat IgG_1	purified	-	BD Biosciences (Germany)
Streptavidin	-	HRPO	-	BD Biosciences (Germany)
FACS				
α-mouse IgG	rat IgG_1	PE	-	BD Biosciences (Germany)
α-rat IgG	goat Ig	FITC	polyclonal	BD Biosciences (Germany)
α-rat IgG	goat	PE	-	BD Biosciences (Germany)
CD4	rat IgG_{2a}, κ	APC	RM4-5	BD Biosciences (Germany)
CD4	rat IgG_{2b}, κ	Biotin	GK1.5	BD Biosciences (Germany)
CD4	rat IgG_{2a}, κ	PE-Cy7	RM4-5	eBioscience (Germany)
CD4	rat IgG_{2b}, κ	FITC	GK1.5	BD Biosciences (Germany)
CD4	rat IgG_{2b}, κ	PE	GK1.5	BD Biosciences (Germany)
CD8	rat IgG_{2a}, κ	PE	53-6.7	BD Biosciences (Germany)
CD8	rat IgG_{2a}, κ	PE-Cy5	53-6.7	eBioscience (Germany)
CD11c	A. hamster IgG1, λ2	APC	HL3	BD Biosciences (Germany)
CD16/CD32	rat IgG_{2b}, κ	purified	2.4G2	BD Biosciences (Germany)
CD25	rat IgG_1, λ	PerCP-Cy5.5	PC61.5	eBioscience (Germany)

Specificity	Isotype	Conjugation	Clone	Source
FACS				
CD69	A. hamster IgG_1, λ	Biotin	H1.2F3	BD Biosciences (Germany)
CD69	A. hamster IgG_1, λ	PE	H1.2F3	BD Biosciences (Germany)
CD90.1 (Thy1.1)	mouse IgG_{2a}, κ	PE-Cy7	HIS51	eBioscience (Germany)
HCV Core	mouse IgG_1	purified	C7-50	Thermo Fisher Scientific (Germany)
HCV NS3	mouse IgG_1	purified	20-8	Thermo Fisher Scientific (Germany)
IFNγ	rat IgG_1, κ	PE	XMG1.2	BD Biosciences (Germany)
ChromePure IgG	rat	purified	-	Jackson ImmunoResearch (United Kingdom)
TNFα	rat IgG_1, κ	APC	MP6-XT22	eBioscience (Germany)
Immunofluorescence microscopy				
α-rat IgG	goat	Alexa594	-	Invitrogen GmbH (Germany)
α-mouse IgG	goat	Alexa488	-	Invitrogen GmbH (Germany)
CD11c	A. hamster IgG	Pacific blue	N418	eBioscience (Germany)
MHC-II	rat IgG_{2b}, κ	Alexa Fluor 700	M5/114.15.2	eBioscience (Germany)
Western Blot				
α-mouse IgG	donkey	HRPO	-	Jackson ImmunoResearch (United Kingdom)
α-rat IgG	goat	HRPO	polyclonal	Dianova (Germany)
HCV Core	mouse IgG_1	purified	C7-50	Thermo Fisher Scientific (Germany)
HCV NS3	mouse IgG_1	purified	20-8	Thermo Fisher Scientific (Germany)
α-OVA	rabbit	HRPO	polyclonal	Quanta BioDesign (USA)

Table 4. Antibodies used for the experiments.

Abbreviations: cluster of differentiation (CD); enzyme-linked immunosorbent assay (ELISA); enzyme-linked immunosorbent spot assay (ELISPOT); fluorescence-activated cell sorting (FACS), horseradish peroxidase (HRPO); Hepatitis C virus (HCV); interferon (IFN); immunoglobuline (Ig); interleukine (IL); ovalbumin (OVA); tumor necrosis factor alpha (TNFα); western blot (WB).

4.1.2 Chemicals and reagents

Chemical agent or reagent	Company
ABTS	Sigma-Aldrich (Germany)
Acrylamide	Carl-Roth GmbH (Germany)
Acetic acid (CH_3COOH)	Merck (Germany)
AEC (substrate kit)	BD Biosciences (Germany)
APS	Sigma-Aldrich (Germany)
BBPcysOVAMPEG	HZI (Germany)
β-Mercaptoethanol	Merck (Germany)
BrefeldinA	Sigma-Aldrich (Germany)
Bromphenol blue	Merck (Germany)
BSA	Sigma-Chemie (Germany)
CD4 T cell isolation kit	Miltenyi Biotec GmbH (Germany)
CD8 T cell isolation kit	Miltenyi Biotec GmbH (Germany)
CFSE	Invitrogen GmbH (Germany)
Citrat acide -1- hydrate ($C_6H_8O_7 \cdot H_2O$)	Riedel-de Haën (Germany)
Collagenase D	Roche Diagnostics GmbH (Germany)
Concanavalin A	Sigma-Aldrich (Germany)
Coomassie R250	SERVA Electrophoresis GmbH (Germany)
CpG	Eurofins MWG Operon (Germany)
Deoxyribonuclease I from bovine pancreas (DNase I)	Sigma-Aldrich (Germany)
Dipotassium phosphate (K_2HPO_4)	Carl-Roth GmbH (Germany)
di-Sodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$)	Carl-Roth GmbH (Germany)
DMF	Sigma-Aldrich (Germany)
DMSO	Sigma-Aldrich (Germany)
Amersham ECL™ Western Blotting Detection Reagents	GE Healthcare (Germany)
EDTA	Sigma-Aldrich (Germany)
FCS	PAA Laboratories GmbH (Germany)
Ficoll-Paque™ PLUS	GE Healthcare (Germany)
Formaldehyde solution 37 %	Sigma-Aldrich (Germany)
L-Glutamin 200 mM	Gibco (Germany)
Glycerol	Carl-Roth GmbH (Germany)
Glycine	Carl-Roth GmbH (Germany)
Guanidine, HCl (GuHCl)	SERVA Electrophoresis GmbH (Germany)
HCV Core Genotype 1b (residues 2-119)	Nordic BioSite (Sweden)

Chemical agent or reagent	Company
HCV NS3-S1b (residues 1192-1459)	Jena Bioscience (Germany)
^{3}[H]-thymidine	GE Healthcare (Germany)
Methanol	J.T.Baker (Europe)
Milk powder	Kaufland (Germany)
Murine GM-CSF	PeproTech EC (United Kingdom)
Heparin sodium 25000	Ratiopharm (Germany)
Hydrogen peroxide (H_2O_2)	Fluka (Switzerland)
IGEPAL® CA-630	Sigma-Aldrich (Germany)
IMDM (GlutaMAX-I)	Gibco (Germany)
Ionomycin	Sigma-Aldrich (Germany)
IPTG	Thermo Fisher Scientific (Germany)
ISF-1 medium	Biochrom AG (Germany)
Isofluran	Delta Select GmbH(Germany)
Isopropanol	J.T.Baker (Europe)
Kanamycin	Sigma-Aldrich (Germany)
Luciferase assay reagent II (LAR II)	Promega GmbH (Germany)
Lysozym	Fluka (Switzerland)
2-MEA	Thermo Fisher Scientific (Germany)
OVA, from chicken egg Grade VII	Sigma-Aldrich (Germany)
EndoGrade OVA (>98 % purity)	Hyglos GmbH (Germany)
$OVA_{323-339}$ peptide ($CD4^+$ OVA-peptide)	HZI (Germany)
H-2_{kb} $OVA_{257-264}$ peptide ($CD8^+$ OVA-peptide; SINFEKL)	HZI (Germany)
PageRuler™ Prestained protein ladder	Fermentas GmbH (Germany)
PAN T cell isolation kit II	Miltenyi Biotec GmbH (Germany)
Penicillin/Streptomycin	Gibco (Germany)
PFA	Carl-Roth GmbH (Germany)
Pierce® Rapid Isotyping Kits – Mouse	Thermo Fisher Scientific (Germany)
PMA	Sigma-Aldrich (Germany)
Poly (I:C)	Invivogen GmbH (Germany)
poly-L-lysine	Sigma-Aldrich (Germany)
Potassium chloride (KCl)	Carl-Roth GmbH (Germany)
Potassium hydrogen carbonate ($KHCO_3$)	Merck (Germany)
Potassium dihydrogen phosphate (KH_2PO_4)	Merck (Germany)
660 nm protein assay reagent	Thermo Fisher Scientific (Germany)
Reflotron® GPT (ALT) test strip	Roche Diagnostics GmbH (Germany)
RPMI 1640 medium (L-glutamine)	Gibco (Germany)

Chemical agent or reagent	Company
Reporter lysis 5x buffer (RLB)	Promega GmbH (Germany)
Scintillation fluid (Betaplate Scint)	PerkinElmer (Germany)
SDS	SERVA Electrophoresis GmbH (Germany)
Sodium acetate (CH_3COONa)	Fluka (Switzerland)
Sodiumdihydrogenphosphate (NaH_2PO_4)	Sigma-Aldrich (Germany)
Sodium carbonate (Na_2CO_3)	Carl-Roth GmbH (Germany)
Sodium chloride (NaCl)	Carl-Roth GmbH (Germany)
Sodium hydrogen carbonate ($NaHCO_3$)	Merck (Germany)
Sodium hydroxide (NaOH)	Carl-Roth GmbH (Germany)
Sulphoric acid (H_2SO_4)	Carl-Roth GmbH (Germany)
Sulfo-SMCC	Thermo Fisher Scientific (Germany)
TCEP	Thermo Fisher Scientific (Germany)
TEMED	Bio-Rad Laboratories GmbH (Germany)
TMB Liquid substrate system	Sigma-Aldrich (Germany)
Triton X-100	Carl-Roth GmbH (Germany)
Tris-Base ($C_4H_{11}NO_3$)	Sigma-Aldrich (Germany)
Tris-HCl ($C_4H_{11}NO_3 \cdot HCl$)	Sigma-Aldrich (Germany)
Trypan blue	Fluka (Switzerland)
Tween 20	Sigma-Aldrich (Germany)

Table 5. Chemical compounds and reagents

Abbreviations: 2,2`-azino-bis (3-ethylbenzothioazoline-6-sulfonic acid) diammonium salt (ABTS); 3-amino-9-ethyl-carbazole (AEC); ammonium persulfate (APS); bovine serum albumin (BSA); cluster of differemtiation (CD); carboxyfluorescein diacetate succinimidyl ester (CFSE); Concanavalin A (ConA); cytosine-phosphate-guanine oligonucleotide sequences (CpG); N,N-Dimethylformamide (DMF); dimethylsulfooxide (DMSO); ethylendieminetetraacetic acid (EDTA); fetal calf serum (FCS); Granulocyte-macrophage colony stimulating factor (GM-CSF); guanidine hydrochloride (GuHCl); Hepatitis C virus (HCV); Helmholtz Centre for Infection Research (HZI); Iscove´s Modified Dulbecco´s Medium (IMDM); Isopropyl-,-D-thiogalactopyranosid (IPTG); 2-mercaptoethylamine HCl (2-MEA); paraformaldehyde (PFA); phorbol 12-myristate 13-acetate (PMA); polyinosine:polycytadilic acid (Poly (I:C)); sodium dodecyl sulfate (SDS); sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (Sulfo-SMCC); tris(2-carboxyethyl)phosphine (TCEP); Tetramethylethylenediamine (TEMED); 3,3′,5,5′-Tetramethylbenzidine (TMB); Tris (hydroxymethyl)-aminomethane (Tris-Base); Tris-(hydroxymethyl)-aminomethanhydrochlorid (Tris-HCl).

4.1.3 Buffers and solutions

Buffer or solution			Composition	
ELISA				
ABTS-solution			0.3 g/l 0.1 M	ABTS citric acid
H_2O_2/ABTS-solution			0.03 %	H_2O_2/ABTS-solution (v/v)
Dilution buffer			1 % 0.1%	BSA/PBS (m/v) Tween 20 (v/v)
2.5 M H_2SO_4-solution			2.5 M	H_2SO_4
Wash buffer			0.1 %	Tween 20/PBS (v/v)
ELISPOT				
0.1 M acetate solution			148 ml 352 ml pH 5.0	0.2 M acetic acid 0.2 M sodium acetate
AEC stock solution			100 mg 10 ml	AEC DMF
AEC substrate solution			333 µl 10 ml 5 µl	AEC stock solution 0.1 M acetate solution 30 % H_2O_2
Blocking solution				RPMI complete
Coating buffer				PBS
Dilution buffer			10 %	FCS/PBS (v/v)
Wash buffer I				H_2O
Wash buffer II			0.1 %	Tween 20/PBS (v/v)
Protein purification				
Core lysis buffer			6 M 10 mM 100 mM pH 8.0	GuHCl Tris-HCl NaH_2PO_4
Core wash buffer	I II III IV	pH 6.3 pH 5.9 pH 5.5 pH 5.0	8 M 10 mM 100 mM pH 6.3 - 5.0	Urea Tris-HCl NaH_2PO_4
NS3 lysis buffer			20 mM 500 mM 1 mg/ml pH 6.3	$Na_2HPO_4 \cdot 2H_2O$ NaCl lysozyme
NS3 wash buffer	I II III	pH 6.3 pH 6.0 pH 5.0	20 mM 500 mM pH 6.3 - 5.0	$Na_2HPO_4 \cdot 2H_2O$ NaCl
NS3 elution buffer			20 mM 500 mM pH 4.0	$Na_2HPO_4 \cdot 2H_2O$ NaCl

Buffer or solution	Composition	
SDS-PAGE		
Coomassie stain	0.1 % 25 % 10 % 65 %	Coomassie R250 (w/v) Isopropanol (v/v) Acetic acid (v/v) H_2O (v/v)
Coomassie decoloration	10 % 40 % 50 %	Acetic acid (v/v) Methanol (v/v) H_2O (v/v)
SDS running buffer	25 mM, pH 8.8 192 mM 0.1 % pH 8.6	Tris-Base Glycin SDS (m/v)
Western Blot		
Antibody dilution buffer (WB)	5 %	Milk powder/TBS-T (m/v)
Blocking buffer (WB)	10 %	Milk powder/TBS-T (m/v)
Blotting buffer (WB)	60 g 29 g 3.7 g adjust to 1 l	Tris-Base Glycin SDS H_2O
10 x TBS	6.1 g 8 g 2 g pH 7.6	Tris-HCl NaCl KCl
TBS-T or wash buffer I (WB)	100 ml 900 ml 0.1 %	10 x TBS H_2O Tween 20 (v/v)
Wash buffer II (WB)	100 ml 900 ml 0.5 M 0.1 %	10 x TBS H_2O NaCl (m/v) Tween 20 (v/v)
Wash buffer III (WB)	100 ml 900 ml 0.1 % 0.5 %	10 x TBS H_2O Tween 20 (v/v) Triton-X 100
Other		
ACK lysis buffer	8.29 g 1 g 0.1 M pH 7.2 - 7.4	NH_4CL $KHCO_3$ EDTA
0.5 M EDTA	0.5 M pH 8.0	EDTA
1% FCS/PBS-solution	1 %	FCS/PBS (v/v)
10 % FCS/PBS-solution	10 %	FCS/PBS (v/v)

Buffer or solution	Composition	
Other		
FACS buffer	2 mM, pH 8.0 2 %	EDTA FCS/PBS (v/v)
Fluorescence conjugation buffer	0.1 M pH 8.5	$NaHCO_3$
4 % Formaldehyde solution	4 %	37 % Formaldehyde solution/PBS (v/v)
1 % Heparin-solution	1 %	Heparin/PBS (v/v)
0.1 % IGEPAL® CA-630	0.1 %	IGEPAL® CA-630/PBS (v/v)
0.9 % NaCl-solution	0.9 %	NaCl/H_2O (m/v)
KH_2PO_4/K_2HPO_4-solution	0.17 M 0.72 M	KH_2PO_4 K_2HPO_4
PBS	136.9 mM 8.1 mM 2.7 mM 1.47 mM pH 7.4	NaCl $Na_2HPO_4 \cdot 2H_2O$ KCl KH_2PO_4
4 % PFA	4 %	PFA/PBS (v/v)
Reporter lysis buffer (RLB)	20 % 0.3 %	RLB/PBS (v/v) BSA
TCEP solution	30 mM 1.5 M (pH 8.8)	TCEP Tris-Base/H_2O (m/v)

Table 6. Composition of used buffers and solutions.

Abbreviations: 2,2`-azino-bis (3-ethylbenzothioazoline-6-sulfonic acid) diammonium salt (ABTS); Ammonium-Chloride-Potassium (ACK); 3-amino-9-ethyl-carbazole (AEC); bovine serum albumin (BSA); N,N-Dimethylformamide (DMF); ethylendieminetetraacetic acid (EDTA); enzyme-linked immunosorbent assay (ELISA); enzyme-linked immunosorbent spot assay (ELISPOT); fluorescence-activated cell sorting (FACS); fetal calf serum (FCS); phosphate buffered saline (PBS); paraformaldehyde (PFA); sodium dodecyl sulfate (SDS); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); Tris buffered saline (TBS); tris(2-carboxyethyl)phosphine (TCEP); western blot (WB).

4.1.4 Bacterial and cell culture media

Medium	Supplements	
BMDC medium	500 ml	RPMI 1640 medium
	10 %	FCS
	1 %	Penicillin/Streptomycin
	1 %	Glutamin
	0.25 mM	β-Mercaptoethanol
IMDM complete	500 ml	IMDM
	10 %	FCS (v/v)
	1 %	Penicillin/Streptomycin (v/v)
	0.25 mM	β-Mercaptoethanol
ISF medium complete	1 l	ISF medium
	1 %	Penicillin/Streptomycin (v/v)
RPMI complete	500 ml	RPMI 1640 medium
	10 %	FCS (v/v)
	1 %	Penicillin/Streptomycin (v/v)
	0.25 mM	β-Mercaptoethanol
TB-medium	12 g	Tryptone
	24 g	Yeast Extract
	4 ml	Glycerin
	900 ml	H_2O
	100 ml	KH_2PO_4/K_2HPO_4-solution

Table 7. Bacterial and cell culture media.

Abbreviations: bone-marrow-derived dendritic cell (BMDC); fetale calf serum (FCS); Iscove´s Modified Dulbecco´s Medium (IMDM); Terrific Broth-medium (TB-medium).

4.1.5 Cell lines

The hybridoma cell lines NLDC-145 and FGK45, producing the rat IgG_{2a} αDEC-205 and rat IgG_{2a} αCD40 respectively, were cultured with ISF medium complete at 37°C and 5 % CO_2.

4.1.6 Expression-ready-clones NS3 and Core

Expression-ready-clones HCV NS3 or HCV Core (BioClone Inc., USA) are synthetic codon-optimized cDNA sequences, encoding a 193 aa HCV NS3 protein (aa 1027-1218) and a 192 aa HCV Core protein (aa 2-191), respectively, and both containing a 6x His-tag at the N-terminus. The synthetic cDNA was cloned between NdeI and XhoI restriction sites of the pET28a expression vector (Novagen, Germany) hosted by the bacteria BL21 (DE3).

4.1.7 Mice

Female Balb/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice were obtained from Harlan Winkelmann GmbH (Borchen, Germany). Thy1.1xOT-I or Thy1.1xOT-II mice on the C57BL/6 genetic background were bred within the animal facility of the Helmholtz Centre for Infection

Research. Thy1.1.xOT-I mice carry transgenic inserts for the mouse TCRα-V2 and TCRβ-V5 genes. This transgenic TCR was designed to recognize OVA peptides (amino acids 257-264; SINFEKL) presented on $H2_{Kb}$. In contrast, transgenic Thy1.1xOT-II mice express the mouse α-chain and β-chain T cell receptor that pairs with the CD4 co-receptor and is specific for OVA peptide residues aa 323-339 in the context of I-Ab. All animals were housed under specific pathogen-free conditions according to the guidelines of the regional animal care committees.

4.1.8 Antigens, adjuvants and peptides

- ***OVA experiments***

For all experiments working with the model antigen OVA, either the OVA Grade VII or EndoGrade OVA (>98 % purity) were used. The immune dominant OVA peptides $CD4_{323-339}$ (ISQAVHAAHAEINEAGR) and $CD8_{257-264}$ (SINFEKL) were synthesized according to established protocols at the HZI and mainly used to re-stimulate activated cells. The adjuvants CpG and Poly (I:C) as well as the synthetic derivate of Mycoplasma macrophage activating lipopeptide-2 (MALP-2), the so called S-[2,3-bispalmitoyiloxy-(2R)-propyl]-R-cysteinyl-amido-monomethoxyl polyethylene glycol (BPPcysMPEG) were used for generating mature DCs. BPPcysOVAMPEG, a compound resulting from coupling BPPcysMPEG to the synthetic peptides encompassing the MHC class I and II restricted OVA peptides ($OVA_{323-339}$, $OVA_{257-264}$), served as a positive control in these experiments. BPPcysMPEG and BPPcysOVAMPEG were synthesized at the HZI and were a kind gift from the Prof. Dr. Carlos Guzmán group (HZI; Department of Vaccinology and applied Microbiology).

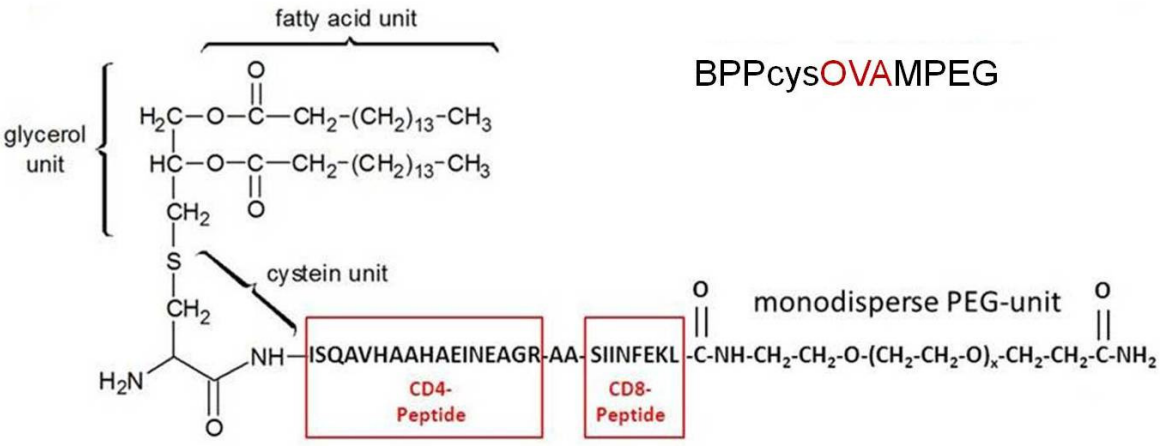

Figure 13. Molecular structure of BPPcysOVAMPEG.

See text for detailed description.

- ***HCV protein experiments***

In the present thesis either the nonstructural protein NS3 or the structural protein Core of the HCV were used since they are the best described ones in the current literature. The purchased NS3 protein contained the immunodominant region aa 1192-1459 (Jena Bioscience, Germany) (solved in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1.5 M urea, 50% glycerol, pH 7.5) and thereby presented the serine protease activity. In contrast, parts of the NTPase/RNA helicase activity were included in the purified form of the NS3 protein (aa 1027-1218; BioClone, USA). The *Escherichia coli (E. coli)* derived recombinant protein containing the HCV nucleocapsid immunodominant region, aa 2-119, was fused with a GST-tag (glutathione S-transferase-tag) at its N-terminus and solved in 1.5 M urea, 25 mM Tris-HCl, pH 8.0 (Nordic BioSite, Sweden). The purified protein comprised the whole sequence of the HCV Core (aa 1-191). In the respective experiments, the adjuvants CpG and Poly (I:C) or αCD40 and Poly (I:C) were used to mature DCs.

The generation of αDEC-205/protein conjugates is described below (4.2.2.4). Both antigens and αDEC-205/protein conjugates were tested for LPS contamination using the HEK-Blue-LPS detection kit (InvivoGen) according to the manufacturer´s instruction.

4.1.9 Recombinant adenovirus

The recombinant adenoviruses Ad-GFP-luc and AdOVA-GFP-luc were originally generated by the group of Prof. Dr. Percy Knolle at the Institute of Experimental Immunology in Bonn and were a kind gift. The E1-deleted and E3-deleted adenoviral vectors (AdLGO) expressing both fusion proteins of the enhanced green fluorescent protein (EGFP) (Promega, Germany) and the click-beetle luciferase (Promega, Germany). In contrast, only AdOVA-luc-GFP additionally expresses the H-2_{Kb}-binding peptide epitope of $OVA_{257-264}$ ($CD8^+$ OVA peptide; SIINFEKL).

Name	Expression of
Ad-GFP-luc	GFP, luciferase
AdOVA-GFP-luc	GFP, luciferase, CD8 OVA peptide

Table 8. Recombinant adenoviruses used for mouse infection.

Abbreviations: adenovirus (Ad); cluster of differentiation (CD); green fluorescent protein (GFP); ovalbumin (OVA).

4.2 Methods

4.2.1 Mouse techniques

Animal experiments were performed according to national and institutional guidelines (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit or the European Council directive 86/609/EEC).

4.2.1.1 Immunization

Groups of female 6 to 10 weeks old C57BL/6 mice (n = 5) were subcutaneously (s.c.) immunized in the lower region of the back on days 0, 14 and 28. 30 µg of both αDEC-205/OVA and DEC-205 antibody alone co-administered to 50 µg Poly (I:C) and 50 µg CpG were injected in a final volume of 50 µl/mouse. In order to inject equal molar amounts of soluble OVA antigen, it was assumed that at least one OVA protein was conjugated to αDEC-205 resulting in aggregates of ~190 kDa in size (4.25:1 mass ratio of αDEC-205 to OVA). On this basis, 7 µg OVA (in 50 µl/mouse) were injected with or without Poly (I:C) and CpG. To compare DEC-205 targeting with the synthetic derivate of MALP-2, animals received either 10 µg BPPcysOVAMPEG or 7 µg OVA, co-administered with 10 µg BPPcysMPEG (50 µl/mouse) as established in the laboratory of Prof. Dr. Carlos Guzmán (HZI, Department of Vaccinology and applied Microbiology).

For HCV experiments, only 5 µg of αDEC-205/Core or αDEC-205/NS3 in a final volume of 30 µl/mouse could be injected s.c. in the hind footpads of Balb/c mice as a consequence of low conjugate concentration. The mice were immunized either with the same concentration of the conjugate (5 µg HCV NS3) thereby representing a several fold higher absolute amount of the antigen or with the calculated conjugated amount of the protein (1.25 µg HCV Core).

4.2.1.2 Sample collection

- ***Isolation of splenocytes***

To obtain a single-cell suspension the spleens were streaked through 100 µm cell strainers (Nylon, BD Biosciences, USA) using the rubber end of a 1 ml syringe plunger (Braun) in a volume of 5 ml PBS. After centrifugation (5 minutes, 350 x g and 4°C), samples were incubated in ACK lysis buffer for one minute to remove erythrocytes. Subsequently the splenocytes were diluted with PBS, filtered through a 100 µm cell strainer and resuspended in buffer or medium after collecting them by centrifugation.

- ***Isolation of lymphocytes***

The lymph nodes (popliteal, inguinal, liver-draining) were mashed through a 100 µm cell strainer in a volume of 1 ml PBS. The resulting single cell suspension was pelleted and resuspended in buffer or medium.

- ***Blood***

Blood samples were obtained by two different routes depending on the experiment.
To monitor the humoral immune response via antigen-specific IgG titer individual blood samples were taken one day before the first (day -1), second (day 13) and third (day 27) immunization as well as 14 days after the last antigen injection. For this, the mice were lightly anaesthized through isofluorane inhalation and 75 µl of blood were collected by penetrating the retro-orbital sinus with a haematocrit capillary (Hirschmann Laborgeräte GmbH & Co.KG, Germany). Sera were taken by allowing the blood to coagulate during 45 minute incubation at 37°C and 5 % CO_2, followed by 45 minutes at 4°C. Then the blood samples were centrifuged at 420 x g for 10 minutes at 4°C to separate the serum from other blood components. The supernatants were carefully removed and stored at -20°C for ELISA.
The alanin aminotransferase (ALT) levels were determined in sera of three times immunized mice before (day 0) as well as after the infection with the particular adenovirus on day 2, 3 and 6. Blood samples were obtained with a sodium heparinized haematocrit capillary (Hirschmann Laborgeräte GmbH & Co.KG, Germany) after puncturing the warmed tail vein and a maximal volume of 75 µl of blood was collected into 25 µl 1 % Heparin-solutions. The tubes were centrifuged (10.600 x g, 10 minutes, room temperature (RT)) and resulting plasma was used for detecting ALT activity.

- ***Liver***

The isolation of liver lymphocytes was always initiated by perfusion of the liver through the left ventricle with a volume of 10 ml ice-cold PBS. The excised organ was minced on ice and finally enzymatically digested using IMDM supplemented with 0.2 mg/ml Collagenase D, 10 µg/ml DNase and 5 % FCS for 30 minutes at 37°C. In the meantime, the livers were pipetted with transfer pipettes (Sarstedt AG & Co., Germany) every 5 minutes to improve tissue disintegration. The digestion was stopped by addition of 0.5 M EDTA to 5 mM final concentration. To remove undigested compounds, the liver suspensions were passed through a 100 µM cell strainer, washed with fresh IMDM and liver lymphocytes were subsequently isolated by Ficoll density gradient centrifugation.

4.2.1.3 Health control

The health status of immunized mice and development of acute side effects potentially induced by the protein-antigen conjugates or adjuvants were monitored. For this, the optical appearance, the behavior, the weight and the body temperature were controlled before the first immunization and one, three and five days after every following immunization.

4.2.1.4 Intravenous infection with recombinant adenoviruses

Immunized mice (as described above) were infected intravenously (i.v.) with the respective recombinant adenovirus on day 42 after the first antigen injection. A dose of $2x10^8$ plaque forming units (PFU) per mouse in a maximal volume of 200 µl sterile 0.9 % NaCl-solution was injected to the warmed tail vein using a fine dosage syringe with integrated 0.3x12 mm cannula (Braun Omnican F).

4.2.2 Protein techniques

4.2.2.1 Purification of αDEC-205 or αCD40 from hybridoma cell lines

The Protein G Sepharose®, Fast Flow (Sigma-Aldrich, Germany) was loaded with supernatants from 2 weeks cultured NLDC-145 or FGK45 hybridoma cells. Follwin a wash step with PBS, the antibody was eluted with 0.1 M Glycin pH 3. Fractions were pooled, dialyzed in Slide-A-Lyzer® Dialysis Cassettes, 10K molecular weight cut off (MWCO) (Thermo Fisher Scientific) against PBS at 4°C overnight and concentrated using centrifugation tubes 10.000 MWCO Vivaspin 20 (Sartorius Stedim Biotech GmbH, Germany). The concentration of purified αDEC-205 and αCD40 was determined by spectrophotometry on the basis of the following formula: conc. [mg/ml] = OD_{280}/1.4

4.2.2.2 Expression and purification of HCV-6 x HIS-tagged proteins

While the purification of HCV NS3 protein was performed under native conditions according to a protocol modified from Vishnuvardhan et al., the HCV Core protein was purified under denaturing conditions following a protocol modified from Mihailova and colleagues (Vishnuvardhan et al. 1997; Mihailova et al. 2006). Protein expression was carried out similarly in both expression-ready-clones HCV NS3 or HCV Core hosted by the bacteria BL21 (DE3). In brief, bacterial cells were grown at 37°C in TB-medium supplemented with a final concentration of 100 µg/ml Kanamycin to an OD_{600} of 0.5 and protein expression was induced through addition of 1 mM IPTG for 2 hours at 37°C. Bacteria from the induced culture were centrifuged for 30 minutes at 6.400 x g at room RT.

- ***NS3***

The cell pellet was resuspended in NS3 lysis buffer (3 ml per 1 g wet weight), incubated for 30 minutes on ice and finally disrupted by using a french press. To remove cell debris, the homogenate was centrifuged (30 minutes, 30.000 x g, 4°C) and then the supernatants were loaded onto a Ni-NTA agarose column (Quiagen, Germany). The column was extensively washed with a stepwise pH gradient using NS3 wash buffers I – III and the NS3 elution buffer was loaded onto the column to elute the HCV NS3 protein.

- ***Core***

Bacteria were resuspended in Core lysis buffer at a ratio of 1:5 (m/v) and stirred for 1 hour at RT. The lysate was centrifuged at 14.000 x g for 30 minutes at RT to pellet cellular debris. Ni-NTA agarose columns (Quiagen, Germany) were subsequently loaded with the cleared supernatants and mixed gently by shaking on a rotary shaker for 4 hours at RT. After incubation the column was extensively washed with a stepwise pH gradient using Core wash buffers I - IV and the proteins were finally eluted with Core elution buffer.

The resulting protein fractions of both HCV NS3 and HCV Core were pooled, concentrated using centrifugation tubes 10.000 MWCO Vivaspin 20 (Sartorius Stedim Biotech GmbH, Germany) and verified by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The protein concentration was calculated using the 660 nm protein assay reagent by means of a BSA standard series.

4.2.2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Protein production and purification steps of HCV proteins as well as DEC-205 antibody were analyzed by SDS-PAGE. The fractions collected during the purification procedure were diluted in 4 x SDS-loading buffer and loaded onto a 15 % SDS-PAGE gel. After running, the protein content in the fractions was detected by Coomassie-stain.

4.2.2.4 Protein conjugation to DEC-205 antibody

In order to establish the conjugation conditions for the DEC-205 antibody to an antigen, different coupling protocols were tested using OVA as model antigen. Besides a protocol initially established by Silvia Prettin (HZI) for fluorescence labeling of antibodies (“fluorescence” protocol), the αDEC-205 conjugation strategy according to the dissertation of Volker Storn (“Storn” protocol), a protocol published by Mahnke et al. (“Mahnke” protocol) and a modified conjugation protocol recommended by the Thermo Fisher technical support (“modified” protocol) were tested (Dissertation Storn 2008; Mahnke et al. 2003; Thermo Fisher Scientific, Germany). Successful conjugation was mainly evaluated by

spectrophotometry and western blot analysis. The conjugation of the HCV proteins to αDEC-205 was performed according to the prior established coupling protocol.

- ***"fluorescence" protocol***

The DEC-205 antibody was diluted to a concentration of 1 mg/ml with the fluorescence conjugation buffer (0.1 M NaHCO3, pH 8.5) and dialyzed overnight at 4°C against the same buffer using a Slide-A-Lyzer Dialysis Cassettes, 10K MWCO (Thermo Fisher Scientific, Germany). Subsequently, the OVA protein was dissolved in DMSO (final concentration 1 mg/ml) and 100 µl of the protein solution were given to 1 ml αDEC-205 (protein to antibody ratio 1:10). The antibody/antigen mixture was incubated for 1 hour at RT utilizing a Thermomixer (Eppendorf Vertrieb Deutschland GmbH). Subsequently, the αDEC-205/OVA batch was dialyzed as described above, but against PBS and for 48 hours at RT in the dark.

- ***"Mahnke" protocol***

In order to chemically activate the protein for conjugation to αDEC-205, the heterobifunctional crosslinker sulfo-SMCC was used. Based on the protocol published by Mahnke et al. the protein was incubated with sulfo-SMCC to allow reaction of the amines of the antibody with the NHS ester of the crosslinker (Mahnke et al. 2003). For this, the antibody (4 mg/ml) was reduced using 2-MEA, which is a mild reductant that is often used to selectively reduce hinge-region disulfide bonds of an antibody resulting in monovalent antibody fragments. The αDEC-205/2-MEA mixture was incubated for 1.5 hours at 37°C and subsequently separated using Zeba Desalt Spin Columns (Thermo Fisher Scientific, Germany). In the meantime, the OVA protein was as well activated with the sulfo-SMCC at 37°C for 30 minutes and excess of the crosslinker was removed by Zeba Desalt Spin Columns (Thermo Fisher Scientific, Germany). Finally, sulfo-SMCC-activated OVA was given to the reduced antibody and incubated overnight at 4°C.

- ***"modified" protocol***

In contrast to the "Mahnke" protocol, not the protein but the DEC-205 antibody was chemically activated by sulfo-SMCC for the conjugation. The amine-reactive NHS ester (N-hydroxysuccinimide ester) of sulfo-SMCC reacts first with the primary amines of the antibody and is then given to the sulfhydryl-containing protein. To expose the sulfhydryl-groups of the proteins, these were first incubated for 1.5 hours at RT in 30 mM TCEP solution. In the meantime, αDEC-205 was activated with the crosslinker at 37°C for 30 minutes. Excess of TCEP and sulfo-SMCC in the respective samples was removed using Zeba Desalt Spin Columns according to the manufacturer´s protocol (Thermo Fisher Scientific, Germany). The reduced proteins were immediately mixed with the activated antibody and incubated overnight at 4°C (Fig. 14). αDEC-205/protein conjugates were separated from free protein

using a column loaded with protein G resin (Thermo Fisher Scientific, Germany) in the first instance. As a consequence of loosing high amounts of protein G purified conjugate, this strategy was later changed to 150 K MWCO Pierce® concentrators (Thermo Fisher Scientific, Germany), which facilitated not only removal of unbound protein, but also resulted in concentration of the sample.

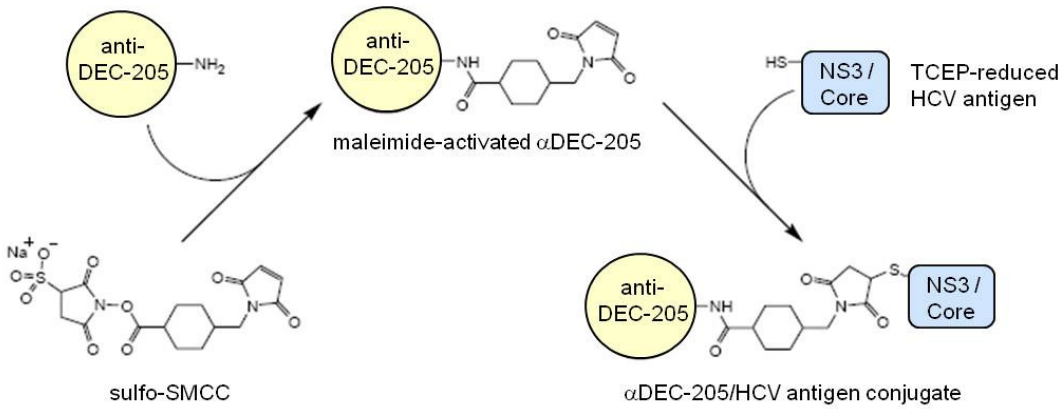

Figure 14. Model of coupling αDEC-205 to proteins.

The NHS ester of sulfo-SMCC initially reacts with the primary amine of the DEC-205 antibody. In a second step the resulting maleimide-activated αDEC-205 reacts with the TCEP-reduced sulfhydryl-group of the HCV antigen. The final product is the chemically coupled αDEC-205/HCV antigen conjugate. Abbreviations: N-hydroxysuccinimide ester (NHS ester); sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC); tris(2-carboxyethyl)phosphine (TCEP).

- ***"Storn" protocol***

The "Storn" protocol differs from the "modified" protocol mainly in two fundamental points. First, the protein was not reduced using TCEP and second, the reactivity of the NHS ester of the crosslinker was tested. For this, sulfo-SMCC was diluted in PBS and 500 µl of the solution was spectrophotometric analyzed (OD_{280}). Subsequently, 100 µl 1 M NaOH was added, mixed for 30 seconds and again spectrophotometric analyzed (OD_{280}). The measured values should increase (60 – 100 %) from the first to the second measurement in order to verify the reactivity of the NHS ester. In the following, 2 mg crosslinker was solved in 200 µl PBS at 50°C using a Thermomixer (Eppendorf Vertrieb Deutschland GmbH). Subsequently, 14 µl of the sulfo-SMCC solution was added to 1 mg αDEC-205 diluted in 300 µl PBS and incubated for 1 to 2 hours on ice. The excess of the cross-linker was removed as described above. The sulfo-SMCC-activated antibody was added to 1 mg protein solved in 300 µl PBS on ice and incubated again for 1 to 2 hours at 4°C.

4.2.2.5 Western Blot

Successful conjugation was verified among others with western blot analysis. In brief, 10 - 12 % SDS-polyacrylamide gels run with samples of the αDEC-205/protein conjugates were

blotted onto 10 % methanol activated polyvinylidene difluoride (PVDF) membranes in blotting buffer for 75 minutes at RT and 125 mA. After an incubation of the membranes in blocking buffer for 1 hour at RT, these were probed with protein-specific antibodies. For OVA-protein detection α-OVA-HRPO was added to the membranes. Either the [20-8] antibody or the [C7-50] antibody was used to detect HCV NS3 or rather HCV Core protein using a secondary donkey α-mouse HRPO-labeled antibody. Staining with goat α-rat IgG conjugated with HRPO was performed to detect only the DEC-205 antibody in αDEC-205/protein conjugates samples. All antibodies were incubated for 45 minutes at RT on the membranes and were removed by three consecutive washing steps. Blots were developed with Amersham ECL™ Western Blotting Detection Reagents.

4.2.2.6 Verification of the αDEC-205/HCV protein conjugate via enzyme-linked immunosorbent assay

In addition to western blot analysis, the ELISA method was utilized to confirm the successful binding of HCV-protein to the DEC-205 antibody.

1 ng/µl of appropriate HCV protein antibody was coated on a 96-well Nunc-Immuno MaxiSorp plate (Nunc., Germany) and incubated overnight at 4°C. Addition of 10 % FCS/PBS-solution stopped the reaction and the plate was washed with PBS using an ELISA-washer (Tecan Deutschland GmbH, Germany). Then the αDEC-205/HCV protein conjugate was added to the plate and after removal of unbound conjugate, donkey α-rat IgG was added to detect the αDEC-205 part of the bound conjugate. The reaction was visualized using the TMB Liquid substrate system and 2.5 M H_2SO_4-solution. The absorbance was detected at a wavelength of 450 nm using an ELISA reader.

4.2.3 Assessment of the functionality of the αDEC-205/HCV protein conjugates

The ability of the αDEC-205/protein conjugate to actually bind to BMDCs *in vitro* was examined with either immunofluorescence microscopy or FACS analysis.

4.2.3.1 Generation of murine bone-marrow derived cells

For the generation of BMDCs, bone marrow cells were isolated from the hind legs of Balb/c mice. Briefly, the skin and the muscles were removed and the legs were disconnected from the body at the hip. After cutting both ends of femur and tibia, the marrow was flushed with 1% FCS/PBS-solution using a syringe with a 0.45 x 0.12 mm needle (Braun). The bone marrow suspension was vigorously resuspended, centrifuged (8 minutes, 350 x g, 4°C) and shortly treated with ACK lysis buffer. Subsequently, the cells were filtered through a 40 µM cell strainer (Nylon, BD Biosciences, USA) and seeded at $4x10^6$ cells per 100 mm uncoated

petri dish (Nunc., Germany) in a final volume of 10 ml BMDC medium supplemented with 5 ng/ml murine GM-CSF. After incubation at 37°C and 5% CO_2 for 3 days, 10 ml of fresh BMDC medium was added. At day 6 of the cultivation the non-adherent BMDCs were carefully harvested and used for binding analyses.

4.2.3.2 Binding analysis by immunofluorescence microscopy

In order to confirm the BMDC phenotype, the harvested cells were sorted by MHC $II^+/CD11c^+$ FACS analysis performed on a FACSAria II cell sorter (BD Biosciences, USA). The sorted MHC $II^+/CD11c^+$ BMDCs were subsequently stained with either DEC-205 antibody alone, the conjugate or soluble protein with a final concentration of 10 µg/ml for 1 hour at 4°C. After washing with RPMI complete, the stained cells were pipetted to poly-L-lysine prepared coverslips (ϕ 12 mm; Thermo Fisher Scientific, Germany) in 24-well dishes (Nunc., Germany). To investigate the binding ability of the DEC-205 antibody and its internalization, the BMDCs incubated again 30 minutes either at 4°C or 37°C. The cells were subsequently fixed with 4 % PFA, washed and permeabilized with 0.1 % IGEPAL® CA-630. Subsequently, the cells were simultaneously stained with goat α-rat IgG conjugated with Alexa594 to detect the DEC-205 antibody as well as with the primary antibody α-HCV Core [C7-50] followed by secondary goat α-mouse IgG Alexa488 (1 hour, 4°C) to verify the bound HCV protein. After fixing the coverslips on microscopy slides, the binding ability to BMDCs and potential internalization of DEC-205 antibody to BMDCs were analyzed by means of immunofluorescence microscopy.

4.2.3.3 Binding analysis by flow cytometry

- ***native version***

To show the capacity of αDEC-205/NS3 as well as αDEC-205/Core to bind to DCs expressing the DEC-205 receptor, both the conjugates and αDEC-205 alone were added to the generated BMDCs at a final concentration of 10 µg/ml. BMDCs without any addition served as control. Then, the cells were incubated for 1 hour at 4°C and after washing for an additional hour at 4°C. APC-labeled αCD11c was added to the BMDCs to enable gating on $CD11c^+$ cells. In order to confirm binding of αDEC-205/HCV antigen conjugates, the cells were stained with either PE-labeled goat α-rat detecting the αDEC-205 or with α-HCV Core [C7-50] and α-HCV NS3 [20-8] respectively, followed by α-mouse IgG_1 PE to visualize coupled HCV antigen. Finally, the BMDCs were washed, resuspended in 200 µl FACS buffer and analyzed by flow cytometry.

- ***fixed version***

In contrast to the native version of the binding analysis, splenocytes were isolated from the spleen as described above (4.2.1.2) and used for binding analysis under fixed conditions. For this, splenocytes were stained with 10 µg/ml of either αDEC-205, the conjugates or soluble protein (15 minutes, 4°C) and subsequently fixed with 4 % PFA for 10 minutes at RT in the dark. The PFA-solution was removed by two washing steps and the cells were stained with FITC-labeled goat α-rat to detect the αDEC-205. To avoid unspecific binding the BMDCs were blocked with ChromePure IgG for 10 minutes at 4°C followed by a second staining step with $CD11c^+$ antibody. For FACS analysis, the cells were pelleted through centrifugation and resuspended in 200 µl FACS-buffer. The binding capacity of DEC-205 antibody alone or the conjugate was analyzed following gating on the $CD11c^+$-population.

4.2.4 Assessment of humoral and cellular immune responses

4.2.4.1 Enzyme-linked immunosorbent assay

- ***OVA experiments***

Sera from individual immunized mice were assayed for the presence of antigen-specific IgG by ELISA using 96-well Nunc-Immuno MaxiSorp plates (Nunc., Germany) coated overnight at 4°C with 2 µg/ml OVA protein diluted in coating buffer. The coated plates were washed 6 times with wash buffer (ELISA) using an ELISA-washer (ELx405, BioTek). To block unspecific binding sites, 200 µl/well blocking buffer was pipetted and incubated for 2 hours at 37°C. Serial twofold dilutions of serum samples in 3 % BSA/PBS (v/v) ranging from 1:100 to 1:102.400 were added using an ELISA pipetting robot (Precision 2000, BioTek). 3 % BSA in PBS (v/v) served as negative control and was later used to determine the background value. The plates were incubated for 2 hours at 37°C and were subsequently washed as described above. The antibody binding was revealed using biotin-conjugated α-mouse IgG (1 hour, 37°C) followed by the secondary antibody Streptavidin-HRPO (30 minutes, 37°C). To visualize the reactions H_2O_2/ABTS-solution was added to each well after 6 washing steps and finally the absorbance was determined at a wavelength of 405 nm using an ELISA reader. Endpoint titers were expressed as the reciprocal of the last dilution, which provided an optical density that was > 0.1 units above the values of negative controls after 30 minutes of incubation.

- ***HCV experiments***

To detect HCV antigen-specific total IgG, IgG subclass (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3) or IgM antibodies, the ELISA was performed similarily as described in 4.2.2.6. In brief, 96-well Nunc-Immuno MaxiSorp plates (Nunc., Germany) were coated with 2 ng/µl of the appropriate

HCV protein. After stopping the reaction, the plates were washed and stained with serum samples of immunized mice in 1:500 dilutions (1 hour, RT). The procedure was finalized by a second staining step with A4a or Ig subclass-specific antibody (1 hour, RT). TMB Liquid substrate system/2.5 M H_2SO_4-solution visualized the reaction and the absorbance was determined at a wavelength of 450 nm using an ELISA reader.

4.2.4.2 ^{3}[H]-thymidine incorporation assay

The ability of T cells to proliferate after stimulation can be determined by measuring the uptake of radioactive ^{3}[H] thymidine, which is incorporated in the DNA. To perform T cell proliferation assays two different strategies have been pursued: in case of the NS3 experiments, 5 x 10^6 lymphocytes/ml from spleen or lymph nodes of immunized mice were seeded in a 96-well sterile flat-bottom cell culture plate and re-stimulated with 1, 5 and 10 µg/ml HCV NS3 (aa 1192-1459) in a final volume of 200 µl IMDM complete. In contrast, T cells from Core experiments were first isolated via magnetic activated cell sorting (MACS) using a PAN-T cell isolation kit according to the manufacturer´s protocol. To facilitate presentation of HCV antigen, BMDCs were generated as described in 4.2.3.1 and 1.25 x 10^6 cells/ml of these were co-cultured with 10 µg/ml of HCV Core (aa 2-119) at 37°C, 5% CO_2 for 30 minutes. Subsequently, 2.5 x 10^6 cells/ml MACS-isolated T cells and 1.25 x 10^6 cells/ml of irradiated splenocytes were added to antigen-pulsed BMDCs. Medium alone served as negative control in both cases. The plates were incubated at 37°C, 5% CO_2 and after approximately four days 1 µCi/well of ^{3}[H]-thymidine in 50 µl IMDM complete was added for the final 16 hours of incubation. Cells were frozen at -20°C to abrogate growth and harvested on glass fiber filters (PerkinElmer; Germany) using cell harvester (Inotech Kunststoff GmbH; Germany). The filters were soaked with scintillation fluid and dried in a microwave. ^{3}[H]-thymidine incorporation into DNA was measured by γ-scintillation counting (1450 Microbeta, Wallach Trilux) for 60 sec/well.

4.2.4.3 Enzyme-linked immunosorbent spot assay

To determine the amount of IFNγ- and IL-4-secreting splenocytes in immunized mice, ELISPOT assays were performed using kits for the detection of murine IFNγ from eBioscience (Frankfurt, Germany; Mouse IFN gamma ELISPOT Ready-SET-Go!®) and IL-4 from BD Biosciences (Heidelberg, Germany; Mouse IL-4 ELISPOT Set). The flat bottomed 96-well plates with 0.45 µm hydrophobic High Protein Binding Immobilin-P-Membrane were coated with 100 µl/well of IFNγ or IL-4 capture antibody diluted in PBS and stored overnight at 4°C. On the next day 200 µl/well of blocking solution were added to avoid unspecific binding and incubated at least 2 hours at RT. Subsequently, 1 x 10^6 and 5 x 10^5 pooled splenocytes within one group of immunized mice were added to the plate in triplicates. The

cells were cultured with 5 µg/ml OVA-protein or $CD4^+$ or $CD8^+$ OVA-peptide for either 24 hours (IFNγ) or 48 hours (IL-4) at 37°C and 5% CO_2. 5 µg/ml of ConA served as positive control whereas RPMI complete was used to estimate background spots (negative control). After removal of the cells by extensively washing, cytokine secretion was detected by staining with the corresponding biotinylated detection antibody and with Streptavidin-HRPO. Colored spots were developed using 100 µl/well of AEC substrate solution for 5 to 60 minutes. The reaction was stopped by washing with deionized water followed by drying the plates for at least 2 hours at RT in the dark. Colored spots were counted with a CTL ELISPOT reader and analyzed using the ImmunoSpot image analyzer software version 3.2. The results were expressed as spot forming units (SPU) for 1 x 10^6 splenocytes/well and calculated as follows: The spots produced by the negative control (cultured with medium) were substracted from the spots produced by the re-stimulated cells (each well of negative control was substracted from each well with re-stimulated cells; triplicates = factor of 9). Subsequently, the mean for each group was calculated and extended to 1 x 10^6 splenocytes/well, if necessary.

4.2.4.4 *In vivo* cytotoxicity assay

In order to determine, whether DEC-205-mediated immunization lead to the induction of OVA-specific CTLs, an *in vivo* cytotoxicity assay was performed. Target cells loaded with $CD8^+$ OVA-peptide were adoptively transferred into immunized mice 9 days following the last immunization and lysis of target cells was assessed.

For each recipient mouse, 2 x 10^7 splenocytes in IMDM complete were split into two equal parts. One part was pulsed with 1 µg/ml $CD8^+$ OVA-peptide for 30 minutes at room temperature and the remaining unloaded splenocytes served as control. Both parts were extensively washed with IMDM complete (without FCS) and subsequently stained with CFSE. The antigen-loaded cells were labeled with a high concentration (2.5 µM) of CFSE ($CFSE^{high}$ population) whereas the control cells were stained with 0.25 µM of CFSE ($CFSE^{low}$ population) to distinguish the two populations. After 8 minutes of incubation at 37°C and 5 % CO_2, the CFSE staining was stopped by addition of FCS. To remove the remaining CFSE, loaded and unloaded cells were pelleted, washed with PBS and pooled to one fraction. 2 x 10^7 splenocytes in 150 µl were i.v. injected into recipient immunized mice.

At 16 hours after adoptive transfer of target cells, splenocytes were isolated from recipient mice and resuspended in FACS-buffer for FACS analysis. Percent lysis of target cells was calculated by the following formulas:

$r = [\%\ CFSE^{low}\ (\text{of all CFSE-positive}) / \%\ CFSE^{high}\ (\text{of all CFSE-positive})]$

$\%\ \text{lysis} = [1 - (r_{\text{unloaded control}} / r_{\text{antigen-loaded mouse}})] \times 100$

4.2.4.5 Flow Cytometry

Antibodies used for flow cytometric analysis were titrated on mouse splenocytes to obtain optimal working dilutions for each antibody-fluorochrome conjugate. Cells were added to 96-well microtitre plates (Greiner) and stained using antibodies as fluorochrome- or as biotin-conjugates, the latter making a secondary staining step with a Streptavidin-fluorochrome conjugate necessary. For fluorescence color-compensation single stainings were performed. Data were acquired on a BD FACSCanto (BD Biosciences, USA) instrument and further analyzed with FlowJo 9.3.1 software (Tree Star, USA).

- ***extracellular staining***

In order to prove expression of specific surface markers, cells were pelleted through centrifugation (10 min, 350 x g, 4°C) and stained with 100 µl/well of the appropriate antibodies diluted in FACS buffer for 15 minutes, at 4°C in the dark. Subsequently, cells were washed with PBS and a second staining step performed, if necessary. After completion of the staining procedure, cells were resuspended in 100 to 200 µl FACS buffer for flow cytometric analysis.

- ***intracellular staining***

For intracellular cytokine detection cells were initially cultured in IMDM complete and re-stimulated with 10 ng/ml PMA and 1 µg/ml Ionomycin for at least 2 hours at 37°C. In order to block cytokine secretion, Brefeldin A was added for the final 2 hours. Subsequently, the cells were washed with PBS and fixed with 2 % PFA/PBS (v/v) (20 minutes, RT, dark). The 2 % PFA solution was removed and the cells were permeabilized with 100 µl/well 0.1 % IGEPAL for 4 minutes at 4°C. Finally, the cells were stained with 100 µl/well of the appropriate antibodies diluted in FACS buffer for 30 minutes and after washing resuspended in 100 to 200 µl FACS buffer for immediate acquisition.

4.2.4.6 Adoptive transfer of Thy1.1/OVA-specific cells into immunized mice

The isolation of OVA-specific OT-I or OT-II cells from Thy1.1xOT-I or Thy1.1xOT-II mice was performed using the appropriate CD4 or CD8 T cell isolation kit by means of MACS. Isolation was carried out according to the manufacturer´s protocol.

In brief, lymphocytes from popliteal, inguinal, liver-draining, mesenteric, axillary and cervical lymph nodes as well as spleens were isolated as described above and resuspended in FACS buffer. For negative isolation, all cells except for the CD4 or CD8 T lymphocytes were initially stained with a Biotin-coupled antibody cocktail followed by binding of anti-biotin MicroBeads. Subsequently, the CD4 or CD8 cells were separated using AutoMACS device (Miltenyi Biotec GmbH, Germany) with the “deplete” program. After confirming the purity and content

of the remaining OVA-specific cells by flow cytometry, the cells were counted and then stained with 2.5 µM CFSE as described above. The resulting CFSE-labeled CD4 or CD8 T lymphocytes were resuspended in sterile PBS and at least 5 x 10^6 antigen-specific cells were injected i.v. into recipient mice in a volume of 150 µl.
24 hours later the recipient mice were immunized s.c. in the hind footpads and after further 3 days the proliferative capacity of transferred cells was analyzed by flow cytometry.

4.2.4.7 Determination of serum alanine transaminase

The serum ALT levels of immunized and recombinant adenovirus infected mice were determined to quantify hepatocyte damage in the animals. A volume of 32 µl plasma (prepared as described above) was pipetted on a Reflotron® test strip and ALT activity was determined using the scil Reflovet® Plus reflection-photometer (scil animal care company GmbH, Germany).

4.2.4.8 Bioluminescence measurement

The enzymatic activity of luciferase in the liver of immunized and recombinant adenovirus infected mice was determined. For this purpose, the selected right, lower lob of the organ was separated into two parts, weighted and fragments were homogenized in proportional volumes of Reporter Lysis Buffer using Lysing Matrix D (MP Biomedicals, Land). A FastPrep-24 (MP Biomedicals) was used 3 times for 10 seconds at 5.5 m/sec to disrupt the tissue. After centrifugation (3 minutes, 10.000 x g, 4°C) the lysates were mixed with Luciferase Assay Reagent II (Promega) at a ratio of 1:5. Measurement of the two separate liver samples from one mouse was performed at two independent time points in a luminometer (Berthold Technologies, Germany). Data of luciferase expression was expressed in relative light units (RLU).

4.2.4.9 Histological analysis of liver tissue

In order to assess the liver damage after adenovirus infection, the organs were sent to Prof. Dr. Achim D. Gruber, Dr. Sophie Bader and Dr. Dorthe von Smolinski from the Institute for Animal Pathology at the Freie Universität Berlin for histology. In brief, mice were sacrificed by CO_2 inhalation and the gall bladder was extracted after rinsing the liver by means of heart puncture with ice-cold PBS. The left, upper lobe of the liver was selected and fixed in either 4 % PFA or 4 % formaldehyde solution for histology.

4.2.5 Statistical analyses

Experimental results were statistically analyzed by the indicated *t*-tests (two-tailed) using the Graph Pad Prism 5 software (Graph Pad software, La Jolla). All data are presented as mean ± SEM and a *p*-value below 0.05 was considered significant.

5 Results

5.1 Part I

Characterization of antiviral immunity in the liver following DEC-205- versus Toll-like receptor 2/6-mediated antigen delivery to dendritic cells

The *in vivo* DEC-205 targeting strategy represents a well-established method for the effective induction of antigen-specific cellular and humoral immune responses (Bonifaz et al. 2002; Bonifaz et al. 2004; Boscardin et al. 2006; Trumpfheller et al. 2006) and has been proven to be a promising strategy for the development of successful immunotherapies (2.3.3.1) (Tacken et al. 2007). Next to DEC-205 targeting, several studies revealed that the TLR2/6 heterodimer agonist MALP-2 or its synthetic derivative has the potential to target antigens not only to macrophages, but also to DCs resulting in enhanced humoral and cellular antigen-specific immune responses after mucosal delivery (Rharbaoui et al. 2002; Borsutzky et al. 2003; Link et al. 2004; Rharbaoui et al. 2004). Thus, MALP-2 and its derivative are also considered to be promising candidate molecules for the development of immune therapeutic or prophylactic interventions and has been successfully probed in various approaches including wound-healing (Niebuhr et al. 2008), vaccination (Borsutzky et al. 2006), tumor therapy (Schneider et al. 2004), infection (Reppe et al. 2009) and treatment of airway inflammation (Weigt et al. 2005).

In order to establish the experimental basis for the intended development of a DC-based vaccine against HCV infection, the first part of this thesis focused on the comparison of the two different targeting strategies with regard to their capacity to induce antiviral immunity in the liver. For this purpose, a conjugate consisting of an antibody specific for DEC-205 (αDEC-205) and the model antigen OVA was produced and utilized for initial vaccination trials. Based on the results obtained with the model antigen, studies were extended to HCV-related protein antigens NS3 and Core in the second part of this thesis.

5.1.1 Chemical conjugation of antigen to the targeting antibody αDEC-205

Several alternative approaches to chemically link an antigen to the DEC-205 antibody have been described in the literature (Bonifaz et al. 2002; Mahnke et al. 2003, Bonifaz et al. 2004). In order to establish the conjugation conditions for the intended development of an HCV vaccine different coupling protocols were tested using OVA as model antigen. Besides a protocol initially established by Silvia Prettin (HZI) for fluorescence labeling of antibodies ("fluorescence" protocol), the αDEC-205 conjugation strategy according to the dissertation of

Volker Storn (“Storn” protocol), a protocol published by Mahnke et al. (“Mahnke” protocol) and a modified conjugation protocol recommended by the Thermo Fisher technical support (“modified” protocol) were tested (4.2.2.4) (Dissertation Storn 2008; Mahnke et al. 2003; Thermo Fisher Scientific, Germany). In every case, the conjugation efficiency was controlled by Western Blot analysis. Successful conjugation was indicated by the detection of the antibody/antigen conjugate (~190 kDa) consisting of αDEC-205 (~144 kDa) and at least one OVA protein (~44 kDa). To optimize conjugation efficiency the following parameters were varied: the usage of the chemical crosslinker for the conjugation, the kind of reducing agent, the antigen to antibody ratio and the purification methods to remove unbound antigen.

Whereas the ”fluorescence” protocol requires the dilution of the antibody in a specific conjugation buffer (0.1 M NaHCO3, pH 8.5) followed by coupling without any additional crosslinker, the sulfo-SMCC crosslinker was utilized for the other conjugation strategies, also with slight modifications. Based on the “Mahnke” protocol the NHS ester of the crosslinker reacts first with the primary amines of the protein. In a second reaction another part of sulfo-SMCC (maleimide) is linked to the sulfhydryl-groups of the monovalent IgG resulting from the previous reduction with 2-MEA. This differs from both, the “Storn” and the “modified” protocol, in which the antibody and not the protein is activated by the sulfo-SMCC crosslinker for the subsequent conjugation to the protein (Fig. 14). Moreover, whereas in the “modified” protocol the protein is reduced with TCEP to expose its sulfhydryl-groups for the conjugation to the crosslinker-activated IgG, the protein remains unmodified in the “Storn” protocol.

Unexpectedly, neither the “fluorescence” nor the “Storn” protocol resulted in successful conjugation of OVA to αDEC-205 as indicated by the absence of a ~190 kDa protein band in lanes 1 and 6 (Fig. 15A). In addition, the protocol published by Mahnke et al. also turned out to be inefficient, since only minor amounts of the conjugate were detectable in Western Blot analysis (data not shown). Interestingly, only the conjugation carried out on the basis of the “modified” protocol revealed high amounts of αDEC-205/OVA as indicated by the strong of ~190 kDa protein band in Fig. 15A, lane 2. As also shown in Fig. 15A some unconjugated protein was present in the sample, since a second fragment (~44 kDa) was detectable in lane 2. In order to remove the excess of free OVA the αDEC-205/OVA conjugate was purified utilizing a protein G column (lane 4, 5). Due to the fact that this purification method resulted in the loss of considerable amounts of αDEC-205/OVA, it was also tried to remove excess of OVA using 150 K MWCO Pierce® concentrators (Fig. 15B). Indeed, loss of the conjugate was minimized, but this strategy was less efficient in removing the unconjugated OVA protein (Fig. 15B, lane 1-3, compare to lane 5-7 OVA protein) . Since immunization experiments with protein G and concentrator purified αDEC-205/OVA revealed no differences (data not shown), the latter method resulting in higher conjugate concentrations was used in the following. Fig. 15C shows α-OVA-HRPO and α-rat IgG-HRPO staining to

test for the presence of both, the OVA protein and the DEC-205 antibody within the conjugate. The αDEC-205 lacking OVA (lane 1, 4) served as negative control, since it should only be detectable by the α-rat IgG-HRPO and not by the antibody specific for OVA. Indeed, positive staining was only observed in lane 1 and not in lane 4. In addition, successful conjugation could be verified by the detection of ~190 kDa antibody/antigen conjugate by both, the α-rat IgG-HRPO (Fig. 15C, left; lane 2) and α-OVA-HRPO (Fig. 15C, right; lane 5) antibodies.

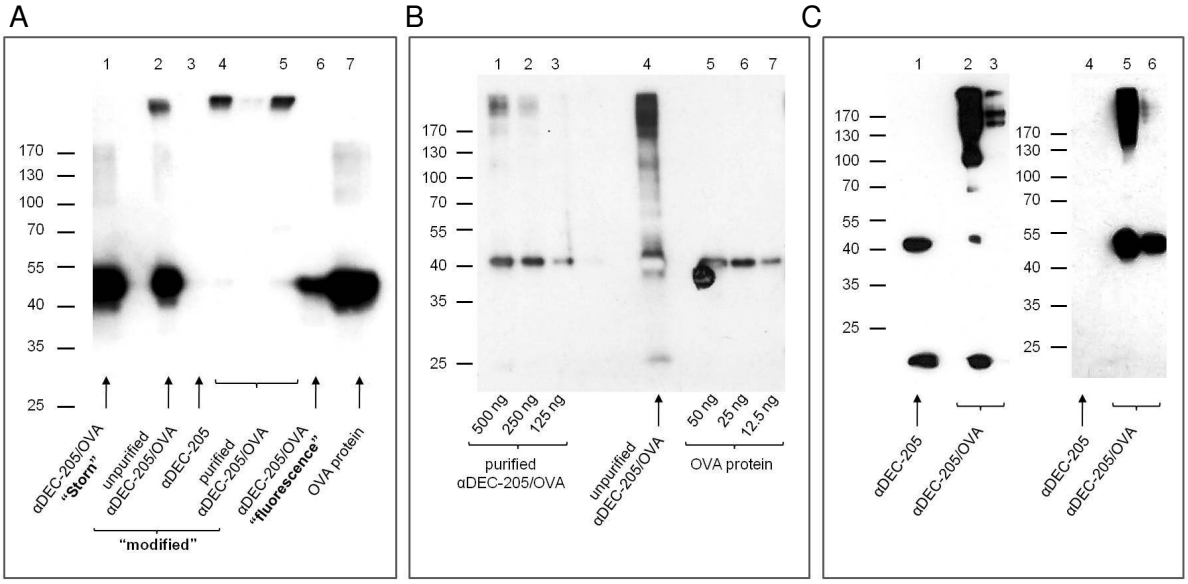

Figure 15. Generation of αDEC-205/OVA conjugate.

In order to analyze conjugation efficiency samples were subjected to SDS-PAGE (10 % polyacrylamide gel) and subsequent Western Blot analysis utilizing α-OVA-HRPO (A, B, C right) or α-rat IgG-HRPO (C left) antibodies. A) Different protocols according to "Storn" (lane 1), "modified" (lane 2, 4, 5) and "fluorescence" (lane 6) were tested to establish efficient conjugation conditions. Lane 4 and 5 display the results obtained for αDEC-205/OVA purification using protein G column in order to remove free OVA protein. B) The excess of unbound protein in the αDEC-205/OVA sample ("modified" protocol) was eliminated by 150 K MWCO Pierce® concentrators (lane 1-3) and the efficiency of this strategy was analyzed. C) Verification of successful αDEC-205/OVA conjugation ("modified" protocol) by staining with either α-rat IgG-HRPO (C left) or α-OVA-HRPO (C right) in order to detect both the αDEC-205 part and the OVA part of the conjugate. Abbreviations: horseradish peroxidase (HRPO); molecular weight cut off (MWCO); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Taken together, the conjugation of OVA to αDEC-205 could be successfully established according to the "modified" protocol, since the coupling based on this strategy resulted in suitable amounts of αDEC-205/OVA for the following immunization trials.

5.1.2 Characterization of adaptive immune responses induced following *in vivo* targeting of antigen to DEC-205 and Toll-like receptor 2/6

Targeting antigens to DCs is an attractive strategy to induce MHC-I- and MHC-II- restricted T cell responses. Recently, a new synthetic derivative of MALP-2, the so called BPPcysMPEG, was successfully used for targeted antigen-delivery to DCs (Prajeeth et al. 2010). Systemic administration of BPPcysMPEG co-administered with OVA resulted in enhanced cross-presentation of the antigen and moreover, in the induction of an effective $CD4^+$ T helper cell-dependent CTL response. In addition, BPPcysOVAMPEG, a construct consisting of the TLR2/6 heterodimer agonist directly linked to the two immunodominant MHC-I and MHC-II OVA peptides (4.1.8), was shown to efficiently prime CTLs towards both antigenic OVA peptides. Therefore, in addition to the αDEC-205 targeting strategy, BPPcysOVAMPEG represents a promising candidate for targeting antigen to DC subsets (Prajeeth et al. 2010).
In order to establish optimal vaccination conditions with respect to inducing HCV-specific immunity, efficiency of DEC-205-mediated targeting of antigen to DCs (αDEC-205/OVA) was compared with vaccination using BPPcysMPEG co-administered with OVA (OVA, BPPcysMPEG) or linked to the immunodominant MHC-I and MHC-II OVA peptides (BPPcysOVAMPEG) Since targeting of antigen to immature DEC-205^+ DCs induces tolerance rather than immunity, it was necessary to overcome the tolerogenic effect by co-administration of TLR ligands to mimic infection and to induce DC maturation (2.3.3.1) (Bonifaz et al. 2002, Bonifaz et al. 2004, Hawiger et al. 2001). To this end, the TLR9 ligand CpG representing a synthetic analogue for bacterial DNA, and the TLR3 ligand Poly (I:C) mimicking double-stranded viral RNA, were used in combination with αDEC-205/OVA for immunization of mice (Napolitani et al. 2005, Zheng et al. 2008, Zhu et al. 2008; Zhu et al. 2010). Strength of the immune response induced using the different *in vivo* DC targeting approaches was monitored by comparative analyses of the OVA-specific humoral (ELISA) and cellular (ELISPOT, cytotoxicity, proliferation) immune responses induced following immunization with special emphasis on the strength of T cell responses induced in the liver draining lymph nodes and liver, the target organ of HCV infection and replication.

5.1.2.1 Analysis of cytotoxic T cell responses in αDEC-205/OVA, OVA, BPPcysMPEG and BPPcysOVAMPEG immunized mice

In order to assess the overall capacity of the αDEC-205/OVA conjugate to induce OVA-specific $CD4^+$ and $CD8^+$ T cell responses *in vivo* an adoptive T cell transfer model was used. To this end, C57BL/6 mice received adoptive transfer of OVA-specific $CD8^+$ or $CD4^+$ T cells isolated from transgenic OT-I and OT-II mice (both $Thy1.1^+$), respectively, followed by s.c. immunization with either αDEC-205 plus Poly (I:C) and CpG (negative control), the αDEC-205/OVA conjugate in addition to the same DC maturation stimuli, OVA co-administered with BPPcysMPEG or the BPPcysOVAMPEG conjugate. On day 3 after transfer the percentage of proliferating $CD4^+/Thy1.1^+$ or $CD8^+/Thy1.1^+$ T cells was determined by FACS analysis and antigen-specific T cell expansion following immunization was calculated on the basis of unspecific proliferation obtained in αDEC-205 immunized mice.

As depicted in Fig. 16 OVA-specific proliferation of $CD8^+$ T lymphocytes was found in all lymph nodes analyzed as well as in the spleen of αDEC-205/OVA immunized mice. Interestingly, this effect was specific for DEC-205 targeting, since BPPcysOVAMPEG immunization and immunization with OVA in conjunction with BPPcysMPEG induced only marginal $CD8^+$ T cell proliferation in all compartments studied with the exception of the popliteal lymph nodes draining the site of injection, where a strong $CD8^+$ T cell expansion was observed following OVA, BPPcysMPEG immunization. Considering the immunological compartments of primary interest with respect to HCV immunotherapy, none of the different vaccination approaches was sufficient to induce $CD8^+$ T cell expansion in the liver, which may be the consequence of the highly tolerogenic environment in this particular tissue (Cyster 1999; Bertolino et al. 2002, Thomson & Knolle 2010). Of note, only in αDEC-205/OVA immunized animals increased percentages of OVA-specific $CD8^+$ T cells were detectable in the liver-draining lymph nodes. Together these data indicate that DEC-205-mediated antigen delivery to DCs is superior in inducing systemic $CD8^+$ T cell activation and expansion in comparison to TLR2/6 based DC targeting strategies and in contrast to BPPcysMPEG either co-administered with soluble OVA or directly linked to the OVA peptides, αDEC-205/OVA induces $CD8^+$ T lymphocyte expansion in the liver-draining lymph nodes, which is of high relevance in the context of HCV liver infections.

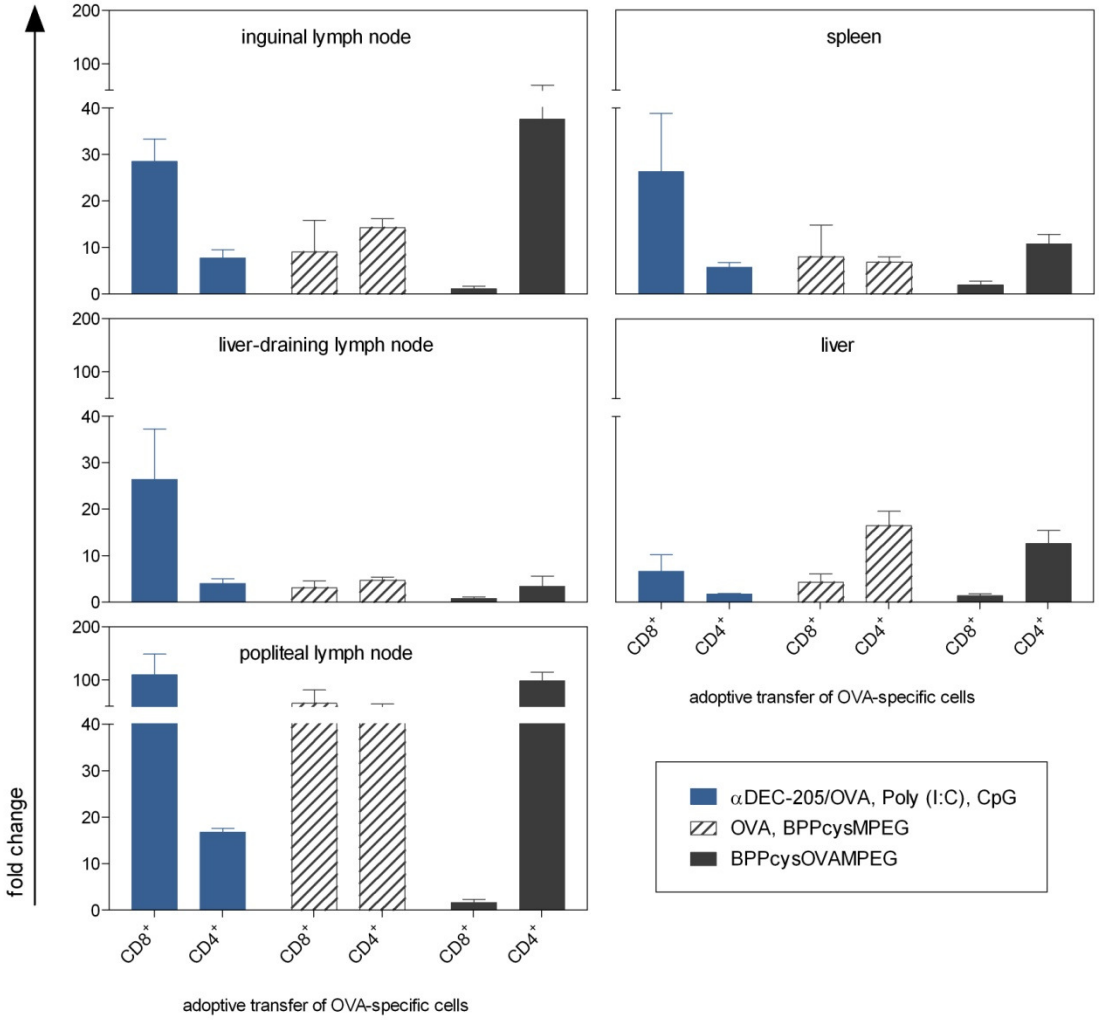

Figure 16. Adoptive transfer of OVA-specific CD4$^+$ or CD8$^+$ T cells into immunized mice.

OVA-specific CD4$^+$ and CD8$^+$ T cells were purified from the spleen and lymph nodes of transgenic OT-I and OT-II mice (both Thy1.1$^+$), respectively by MACS and subsequently labeled with 2.5 µM CFSE dye. 2 x 10^6 OT-I and 1.3 x 10^7 OT-II cells were injected i.v. into recipient C57BL/6 mice. 24 hours later the mice were immunized s.c. in the hind footpad with either 30 µg αDEC-205 or 30 µg of αDEC-205/OVA in addition to 50 µg Poly (I:C) and 50 µg CpG, 7 µg OVA co-administered with 10 µg BPPcysMPEG or 10 µg BPPcysOVAMPEG. 3 days later T cell proliferation in inguinal, liver-draining and popliteal lymph nodes as well as in liver and spleen was evaluated by flow cytometry gating on CD4$^+$ Thy1.1$^+$ or CD8$^+$ Thy1.1$^+$ T cells. The percentages of adoptively transferred Thy1.1$^+$ T cells in immunized recipient mice are depicted as fold change and calculated as follows: Given that αDEC-205 injection did not induce antigen-specific proliferation of transferred T cells, mean percentage of Thy1.1$^+$ T cells recovered from αDEC-205 treated control mice was defined as base value for non-dividing T cells. Fold change of T cell proliferation in immunized mice was calculated by dividing mean percentage of recovered Thy1.1$^+$ T cells of immunized mice through the base value. The bars represent means ± SEM (n = 3) and one representative of two independent experiments is displayed. Abbreviations: carboxyfluorescein diacetate succinimidyl ester (CFSE); magnetic activated cell sorting (MACS); standard error of the mean (SEM).

Considering the $CD4^+$ T cell response, BPPcysOVAMPEG injected mice showed a strong $CD4^+$ T cell proliferative capacity in popliteal and inguinal lymph nodes, which are located close to the application site. Moreover the induced adaptive immune response following BPPcysOVAMPEG injection seemed to be more $CD4^+$ than $CD8^+$ T cell dominated. In contrast, co-administration of the entire OVA protein instead of the immunodominant MHC-I- and MHC-II-restricted OVA peptides (BPPcysOVAMPEG) induced both $CD4^+$ and $CD8^+$ T cell proliferation, although proliferation was limited to the popliteal lymph nodes draining the site of antigen application. In case of αDEC-205/OVA immunization the percentage of $CD4^+$ T lymphocytes recovered from the different compartments was comparatively low suggesting that the DEC-205 targeting strategy is very valid for inducing $CD8^+$ T cell responses, but of limited use for the induction of $CD4^+$ T cell expansion. As for $CD8^+$ T cells, none of the vaccination approaches tested resulted in a statistically significant proliferation of OVA-specific $CD4^+$ T cells in the liver.

Since $CD8^+$ T lymphocytes are of particular importance for effective virus clearance, the extent of antigen-specific $CD8^+$ T cell proliferation in the liver-draining lymph nodes after immunization with αDEC-205/OVA, BPPcysOVAMPEG or OVA in combination with BPPcysMPEG was investigated in more detail. In line with Fig. 16, OVA-specific $CD8^+$ T cells from animals which received αDEC-205/OVA treatment showed almost 100 % proliferation as demonstrated by the complete loss of the CFSE dye. This indicates rapid entry of OVA-specific $CD8^+$ T cells into the cell cycle and that the cells underwent multiple rounds of division, underlining the effectiveness of DEC-205-mediated antigen delivery to DCs with respect to the induction of $CD8^+$ T cell responses (Fig. 17A). Interestingly, although the percentage of proliferating $CD8^+$ T lymphocytes was lower in comparison to αDEC-205/OVA treatment, immunization with OVA co-administered with BPPcysMPEG resulted in antigen-specific proliferation of a considerable number (76.4 %) of the adoptively transferred OVA-specific $CD8^+$ T cells. As expected OVA-specific $CD8^+$ T cells did not respond to αDEC-205 treatment nor did they expand following BPPcysOVAMPEG injection.

Regarding $CD4^+$ T cells, only very few OVA-specific cells were detectable in the liver-draining lymph nodes of all mouse groups tested. Since this was true for every experimental repetition it was difficult to obtain reliable data concerning their overall proliferative capacity in this particular compartment. However, about 85 % of OVA-specific $CD4^+$ T cells underwent proliferation in mice either treated with αDEC-205/OVA or BPPcysOVAMPEG (Fig. 17B). Thus, whereas αDEC-205 targeting of OVA to DCs induced activation of both $CD8^+$ and $CD4^+$ T cells, this was not the case for the BPPcysOVAMPEG construct consisting of the TLR2/6 agonist BPPcysMPEG and the immunodominant MHC-I and MHC-II OVA peptides, which was found to induce strong proliferation of OVA-specific $CD4^+$ but not $CD8^+$ T cells in

the lymph node draining the liver. In addition, the proliferative capacity of OVA-specific $CD4^+$ T cells isolated from OVA, BPPcysMPEG immunized mice was significantly lower than in animals immunized with αDEC-205/OVA and BPPcysOVAMPEG (68.4 %). This is in line with the results obtained for $CD8^+$ T cells (Fig. 17A), suggesting that OVA co-administered with BPPcysMPEG activates both $CD4^+$ and $CD8^+$ T cells, but not to the extent as αDEC-205/OVA does. As expected the control group treated with αDEC-205 alone did not activate $CD4^+$ T cells to enter cell division (Fig. 17B).

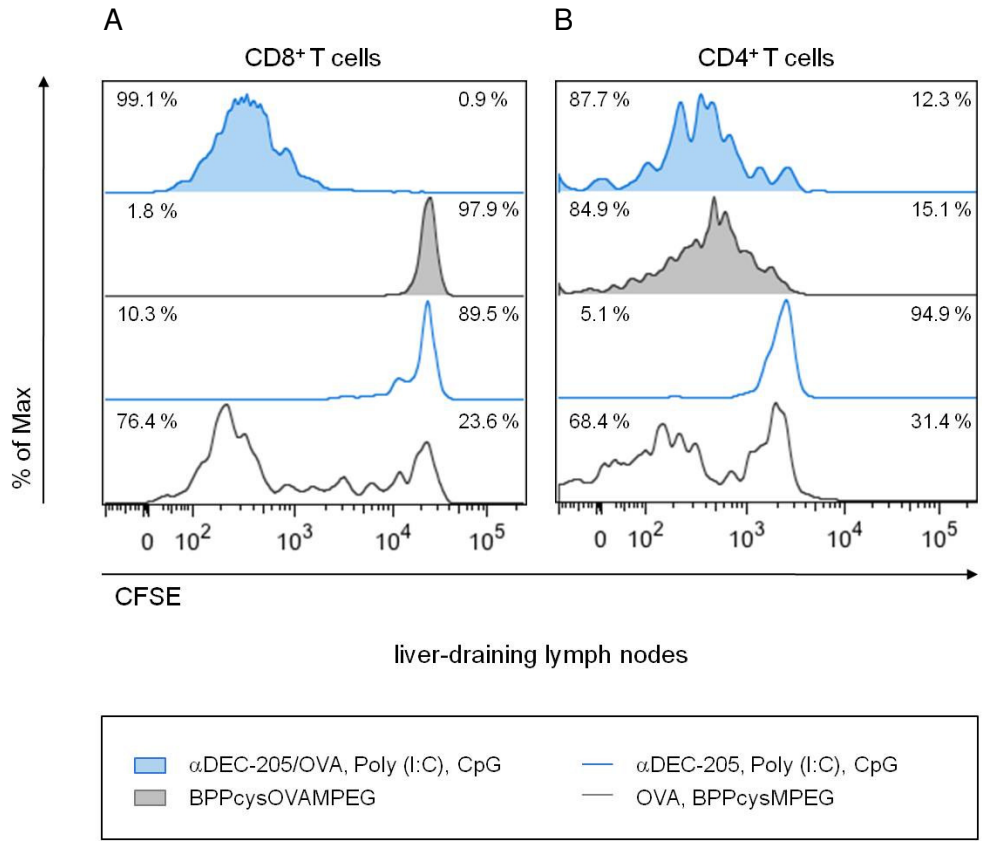

Figure 17. CFSE dilution of $Thy1.1^+$ cells in liver-draining lymph nodes after adoptive transfer of OT-I cells or OT-II cells.

2 x 10^6 CFSE-labeled OVA-specific $CD8^+$ T cells (A) or 1.3 x 10^7 $CD4^+$ T cells (B) isolated from spleen and lymph nodes of OT-I and OT-II mice, respectively, were purified by MACS and injected i.v. into recipient C57BL/6 mice (n = 3). 24 hours later the mice were immunized s.c. in the hind footpad with either 30 µg αDEC-205 or 30 µg αDEC-205/OVA (each in addition to 50 µg Poly (I:C) and 50 µg CpG), 7 µg OVA co-administered with 10 µg BPPcysMPEG or 10 µg BPPcysOVAMPEG. Histograms display the CFSE dilution of $CD8^+$ $Thy1.1^+$ or $CD4^+$ $Thy1.1^+$ T cells in liver-draining lymph nodes of one individual mice (one representative of two independent experiments is shown). Abbreviations: carboxyfluorescein diacetate succinimidyl ester (CFSE); magnetic activated cell sorting (MACS).

5.1.2.2 Cytotoxic T lymphocyte responses in αDEC-205/OVA and BPPcysOVAMPEG immunized mice

The generation of pathogen-specific CTLs plays a crucial role in viral clearance and thus for the development of an effective immunotherapy against viral infections. In order to investigate, to which extend $CD8^+$ T cells primed following αDEC-205/OVA or BPPcysMPEG-based OVA immunization (Fig. 16) (5.1.2.1) would be capable to efficiently kill OVA-bearing target cells, *in vivo* cytotoxicity assays were performed. To this end, C57BL/6 mice were immunized once (on day 0), twice (on day 0, 14) or thrice (on day 0, 14, 28). Cytotoxic activity of OVA-specific $CD8^+$ T lymphocytes following immunization was analyzed 10 days after the last immunization. To this end, splenocytes from untreated animals were isolated, loaded with the MHC-I OVA-peptide (SIINFEKL) or were left untreated and were subsequently labeled with two different concentration of CFSE, resulting in an antigen-positive $CFSE^{high}$ and antigen-negative $CFSE^{low}$ target cell population (Fig. 18A, right upper panel). Following adoptive transfer of equal numbers of both $CFSE^{high}$ and $CFSE^{low}$ splenocytes into immunized recipient mice, the antigen-positive target cell population ($CFSE^{high}$) should be recognized and lysed by OVA-specific CTLs. To assess cytotoxic activity, splenocytes from αDEC-205/OVA, BPPcysOVAMPEG and OVA, BPPcysMPEG immunized mice were isolated 16 hours after transfer of CFSE-labeled target cells and the ratio between $CFSE^{high}$ (antigen-loaded) and $CFSE^{low}$ (antigen-negative) target cells was quantified by flow-cytometry. Percent of lysis as an indicator of cytotoxic activity was calculated on the basis of this ratio (Fig. 18).

Interestingly, αDEC-205/OVA as well as BPPcysOVAMPEG immunization induced priming of OVA-specific CTLs already after a single immunization as indicated by the marked reduction of $CFSE^{high}$ OVA-loaded target cells (Fig. 18A). In more detail, cytotoxic activity was comparatively lower after a single immunization (*10 days*; 56.3 %) than after the second (*24 days;* 81.5 %) and third (*38 days;* 88.6 %) immunization in the αDEC-205/OVA treated group. In contrast to this, BPPcysOVAMPEG immunization resulted in 82.2 % target cell lysis already after the first immunization (*10 days*), even slightly increasing after two immunizations (*24 days;* 88.2 %,) followed by a decline down to 62.6 % specific lysis after the third treatment (*38 days*) (Fig. 18B).

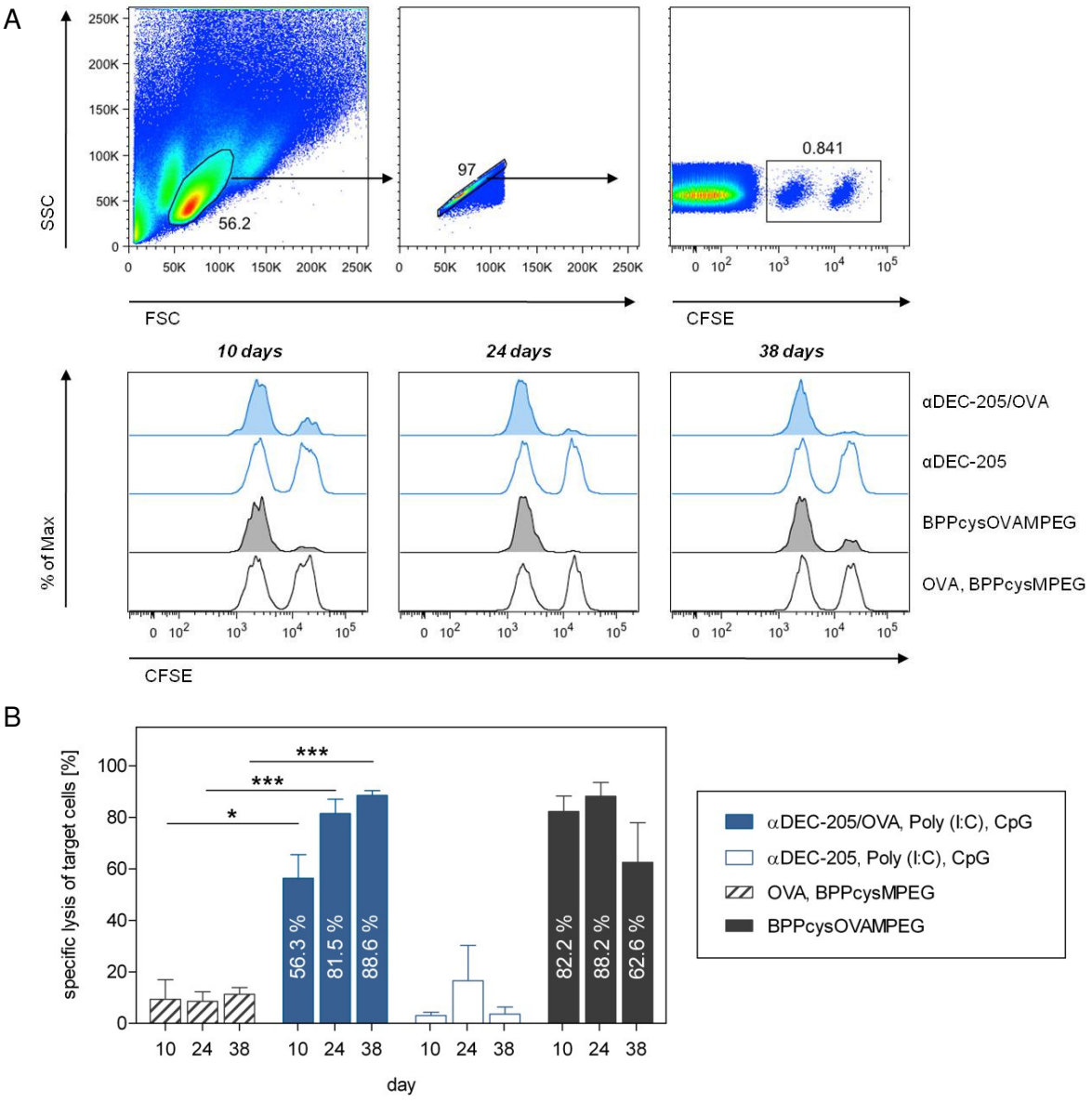

Figure 18. Analysis of OVA-specific cytotoxic T lymphocyte activity following αDEC-205/OVA immunization in comparison to the Toll-like receptor 2/6 targeting strategy.

Mice (n = 3) were s.c. immunized once (on day 0; *10 days*), twice (on days 0, 14; *24 days*) or thrice (on days 0, 14, 28; *38 days*) with either 30 µg αDEC-205 or 30 µg of αDEC-205/OVA (each in addition to 50 µg Poly (I:C) and 50 µg CpG), 7 µg OVA co-administered with 10 µg BPPcysMPEG or 10 µg BPPcysOVAMPEG. 9 days after the last treatment, 1 x 10^7CFSE-labeled splenocytes pulsed with the MHC-I OVA-peptide SIINFEKL ($CFSE^{high}$) or left untreated ($CFSE^{low}$) were injected i.v. into immunized recipient mice to determine CTL-mediated specific lysis by flow-cytometry. A) CFSE-positive splenocytes are depicted as histogram and the $CFSE^{low}$ to $CFSE^{high}$ ratio was determined providing the basis for calculation of OVA-specific lysis. Each histogram displays the results obtained for one out of three individual mice. B) OVA-specific lysis was calculated on the basis of the $CFSE^{low}$ to $CFSE^{high}$ ratio comparing immunized mice and the respective control animal. The bars represent the mean ± SEM (n=3) and were compared by unpaired, two-tailed *t*-test *(* p = 0.0172, *** p < 0.001)*. Abbreviations: carboxyfluorescein diacetate succinimidyl ester (CFSE); standard error of the mean (SEM).

Considering the control animals, which received αDEC-205 without antigen, no target cell lysis was observed as expected. Of note, in mice treated with OVA, BPPcysMPEG no

specific killing of OVA-loaded target cells was detectable independent of the number of immunizations (Fig. 18A).

Taken together, αDEC-205/OVA immunization efficiently induces OVA-specific CTL responses, which was also true for BPPcysOVAMPEG treated mice. In contrast, soluble OVA co-administered with BPPcysMPEG did not result in the induction of CTL responses and specific lysis of OVA-loaded target cells.

5.1.2.3 Characterization of cellular and humoral immune responses following *in vivo* targeting of antigen to DEC-205 vs. Toll-like receptor 2/6 on dendritic cells

Having verified the *in vivo* functionality of αDEC-205/OVA targeting in an adoptive transfer model using OVA-specific $CD4^+$ and $CD8^+$ T cells and additionally in *in vivo* cytotoxicity assays following immunization, the quality of both cellular and humoral immune responses induced by αDEC-205/OVA immunization and in comparison to BPPcysMPEG based *in vivo* DC-targeting was studied in more detail. To this end, a vaccination protocol was used previously established in the lab of Carlos Guzmán, HZI (Ebensen et al. 2007; Libanova et al. 2010; Ebensen et al. 2011). For this, C57BL/6 mice were immunized with αDEC-205/OVA or soluble OVA protein co-administered with Poly (I:C) and CpG. The other groups received BPPcysOVAMPEG and OVA, BPPcysMPEG, respectively. In addition, PBS with or without DC maturation stimuli and αDEC-205 co-administered together with Poly (I:C) and CpG served as internal controls. Since it was found that the Poly (I:C) and CpG as well as BPPcysMPEG treatment did not induce any unspecific immune responses (data not shown), only results obtained from the PBS treated group without any adjuvants are depicted in Fig. 19 and 20. To exclude acute side effects potentially induced by the applied conjugates or adjuvants, mice were monitored with respect to optical appearance, behavior, weight and body temperature before the first injection and on the first, third and fifth day following every further immunization. During the whole time course of the experiment changes in weight as well as in body temperature were found to be in a normal range in all groups of immunized and untreated mice and no abnormalities were detectable (data not shown), indicating that all treatments were well tolerated by the animals and did not induce acute side effects.

- ***Cellular immune responses***

To gain further insights in the kind of adaptive cellular immune response following the different immunization approaches, ELISPOT assays were performed 14 days after the last injection. For this purpose, splenocytes recovered from immunized mice were pooled and cultured 24 (IFNγ) or 48 hours (IL-4) in the presence of the immunodominant MHC-I- and MHC-II-restricted OVA peptides or the entire OVA protein to determine the number of T cells secreting IFNγ or IL-4, indicative for the presence of Th1 or Th2 effector cells, respectively.

As depicted in Fig. 19A the highest number of IFNγ secreting T cells was observed in the BPPcysOVAMPEG immunized group after re-stimulation with both the MHC-I and MHC-II OVA peptide. Interestingly, the number of IFNγ secreting splenocytes from αDEC-205/OVA immunized mice following antigen-specific re-stimulation with the MHC-II OVA peptide was as well significantly increased and reached a level similar to that found after OVA plus Poly (I:C) and CpG immunization, but was significantly higher *(* p = 0.0432)* than in the BPPcysMPEG immunized group (Fig. 19A, upper panel). This effect was even more pronounced for $CD8^+$ T cells (lower panel of Fig. 19A). Here, the number of IFNγ producing splenocytes from αDEC-205/OVA immunized mice was significantly higher than in OVA, Poly (I:C), CpG (**** p = 0.0009*) and OVA, BPPcysMPEG (** p = 0.0435*) immunized groups. Thus, targeting OVA to DEC-205 on DCs is more efficient with respect to the induction of IFNγ secreting effector T cells when compared to immunization using soluble OVA plus adjuvants. However, immunization with BPPcysOVAMPEG induced the highest number of effector T cells secreting IFNγ, a cytokine of great importance in the context of antiviral immunity.

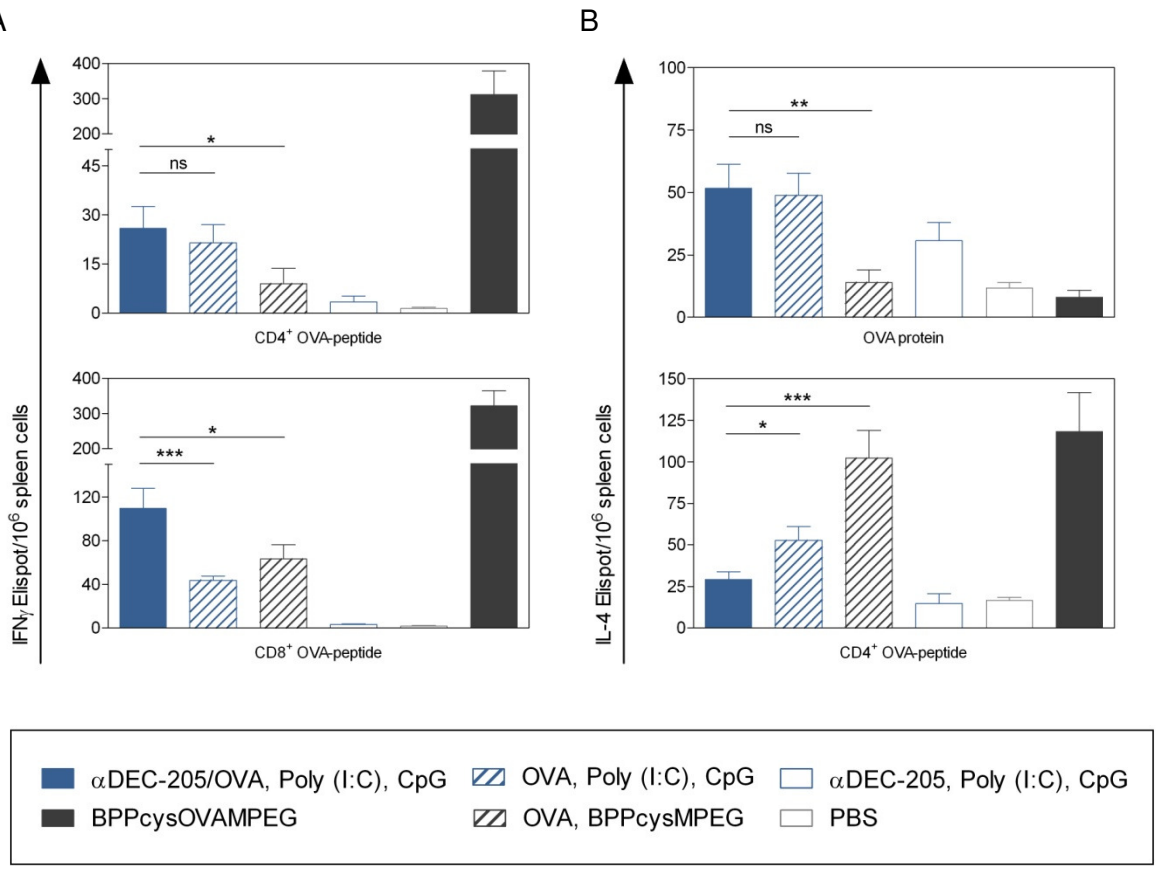

Figure 19. Analysis of the T cell responses stimulated in immunized mice.

ELISPOT assays were performed to quantify the number of cytokine secreting T cells in the spleen of animals after three immunizations (day 0, 14 and 28). Mice were immunized s.c. with either 30 µg αDEC-205/OVA or 30 µg αDEC-205 (in addition to 50 µg Poly (I:C) and 50 µg CpG). The other groups of mice received either 10 µg BPPcysOVAMPEG, 7 µg of soluble OVA protein co-administered together with 50 µg Poly (I:C) and 50 µg CpG or 10 µg BPPcysMPEG. PBS treatment served as control. Cells (1 x 10^6 and 5 x 10^5 cells/well) recovered from the spleen of immunized mice were pooled and incubated for 24 hours (IFNγ) and 48 hours (IL-4) in the presence of the immunodominant MHC-I- and MHC-II-restricted OVA peptides or OVA protein. Then, the number of IFNγ and IL-4 producing T cells was determined. Results are expressed as spot forming units per 10^6 cells. The bars represent the mean ± SEM (n = 5, triplicates from pooled animals) of three independent experiments and were compared by unpaired, two-tailed *t*-test. A) Detection of IFNγ secreting T cells after MHC-II (A upper panel) and MHC-I OVA-peptide (A lower panel) re-stimulation *(* $p < 0.05$, *** $p < 0.001$)*. B) Detection of IL-4 secreting T cells after OVA protein (B upper panel) and MHC-II OVA-peptide (B lower panel) re-stimulation *(* $p = 0.0195$, ** $p = 0.0011$, *** $p < 0.0001$)*. Abbreviations: enzyme-linked immunosorbent spot (ELISPOT); standard error of the mean (SEM).

Next to IFNγ which is a key cytokine of Th1 effector cells, the number of IL-4 secreting Th1 cells was determined (Fig. 19B). Interestingly, re-stimulating splenocytes from BPPcysOVAMPEG immunized mice with the OVA protein did not result in any IL-4 production (19B, upper panel). In contrast, when the immunodominant MHC-II OVA-peptide was used for the re-stimulation (19B, lower panel), the highest number of IL-4 secreting $CD4^+$ T cells were detectable when compared to the other immunization approaches. In case splenocytes were isolated from αDEC-205/OVA immunized mice OVA protein re-stimulation

led to increased IL-4 secretion, which was, however, only slightly higher than in the OVA, Poly (I:C), CpG immunized group. Nevertheless, compared to OVA, BPPcysMPEG immunization, the IL-4 release was significantly higher *(** p = 0.0011)* (Fig. 19B, upper panel). In contrast to this, fewer cells produced the Th2 cytokine in αDEC-205/OVA immunized mice, when splenocytes were incubated with the MHC-II OVA peptide. Interestingly, in case of OVA, Poly (I:C), CpG injection comparable numbers of IL-4 secreting T cells were detectable following re-stimulation with either the OVA protein or the MHC-II OVA peptide. In case of peptide re-stimulation significantly more IL-4 producing T cells were detectable than in the αDEC-205/OVA immunized group *(* p = 0.0195)* (Fig. 19B, lower panel). The same was true for OVA, BPPcysMPEG immunization *(*** p < 0.0001)* (Fig. 19B, lower panel).

In conclusion, αDEC-205/OVA immunization induces a Th1-dominated effector T cell response as indicated by the pronounced IFNγ release after re-stimulation with both immunodominant MHC-I and MHC-II OVA peptides (Fig. 19A). With respect to the TLR2/6 targeting strategy, BPPcysOVAMPEG was found to highly efficiently induce both IFNγ and IL-4 secreting T cells specific for the two immunodominant OVA peptides, which are as well incorporated in the synthetic MALP-2 derivate used for the immunization.

- ***Humoral immune responses:***

Since neutralizing antibodies play an essential role in antiviral immunity, a further important aspect to study was, if and to which extend targeting of antigen to DEC-205$^+$ DCs in comparison to other vaccination strategies would induce OVA-specific antibody responses. To this end, blood samples were collected one day before the first (day 0) and every following immunization (day 13 and day 27) as well as on day 42, when the mice were sacrificed. Sera from every individual mouse were analyzed by ELISA for the presence of antigen-specific IgG antibodies and endpoint titers were determined.

Of note, mice receiving αDEC-205/OVA treatment showed the fastest humoral immune response almost reaching the maximum level already after two injections (Fig. 20A). Whereas in these animals the antigen-specific IgG titer only slightly increased within the next 14 days following the third immunization, the BPPcysOVAMPEG injected mice initially exhibited a weaker IgG response after the first two immunization, which was drastically enhanced after the third boost on day 28.

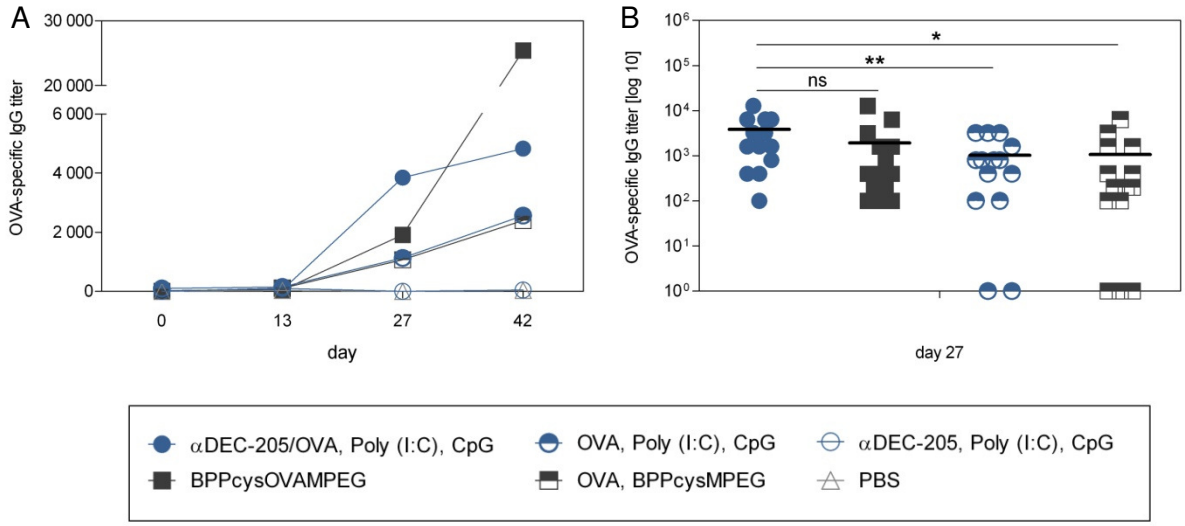

Figure 20. Significant OVA-specific total IgG titer on day 27 after αDEC-205/OVA immunization.

Mice were immunized s.c. on day 0, 14 and 28 with 30 µg αDEC-205/OVA or 30 µg αDEC-205 in addition to the maturation stimuli (50 µg Poly (I:C), 50 µg CpG). The other groups of mice received either 10 µg BPPcysOVAMPEG, 7 µg of soluble OVA protein co-administered with 50 µg Poly (I:C) and 50 µg CpG or 10 µg BPPcysMPEG. PBS treatment served as control. Sera from individual mice were taken the day before every immunization and at the end of the experiment on day 42 and analyzed for the presence of OVA-specific IgG antibodies by ELISA. Results are expressed as endpoint titers of three independent experiments and statistically analyzed using the unpaired, two-tailed *t*-test (n = 5) *(* p = 0.0109, ** p = 0.0070)*. A) Kinetic analysis of OVA-specific total IgG responses in sera from immunized mice. Data were expressed as mean of endpoint titers. B) OVA-specific total IgG titer of individual mice on day 27 after the first immunization (mean ± SEM). Abbreviations: enzyme-linked immunosorbent assay (ELISA); standard error of the mean (SEM).

In addition, animals injected with OVA protein co-administered either with Poly (I:C) and CpG or BPPcysMPEG showed a constant increase in the IgG antibody response over time. However, antibody titers did not reach the level of those found in αDEC-205/OVA or BPPcysOVAMPEG immunized mice at day 27 and 42 post immunization (Fig. 20A). Moreover, at the early stage of the experiment (day 27) this effect was significant when comparing the groups of αDEC-205/OVA immunized mice with mice treated with soluble OVA protein in combination with adjuvant (OVA, Poly (I:C), CpG: ** *p = 0.0070*; OVA, BPPcysMPEG: * *p = 0.0109*) (Fig. 20B).

In summary, αDEC-205/OVA immunization triggers both cellular and humoral immune responses. In more detail, *in vivo* antigen delivery to DCs by targeting the DEC-205 endocytosis receptor on their surface predominantly induces a Th1 effector T cell response as indicated by the pronounced IFNγ release following antigen-specific re-stimulation of splenocytes from αDEC-205/OVA vaccinated mice. In addition to the cellular immune response elevated OVA-specific IgG levels in sera already after the second immunization

(day 27) demonstrate the capacity of αDEC-205/OVA treatment to induce a fast and strong humoral immune response. Moreover, the observed induction of a strong and systemic proliferative capacity of OVA-specific $CD8^+$ T cells, especially in the liver-draining lymph nodes (5.1.2.1), combined with their strong cytotoxic activity (5.1.2.2) underlines that DEC-205-mediated targeting of antigen to DCs represents a valid tool suitable for the establishment of an immunotherapy against HCV.

5.1.3 Comparative analysis of antiviral immunity induced in the liver of αDEC-205/OVA, OVA, BPPcysMPEG and BPPcysOVAMPEG immunized mice

In the previous experiments it was found that targeting antigen to $DEC\text{-}205^+$ DCs induces systemic activation of IFNγ secreting $CD8^+$ T lymphocytes with cytotoxic capacity (5.1.2). To address the question, whether these CTLs would efficiently clear virus infected hepatocytes, an adenovirus challenge approach was utilized to mimic HCV liver infection. To this end, C57BL/6 mice were immunized as described before (5.1.2.3). In order to monitor successful priming of antigen-specific cells, OVA-specific IgG titer in sera were analyzed by ELISA (data not shown). On day 48 after the first immunization animals were infected i.v. with 2×10^8 PFU of the recombinant adenovirus AdOVA-luc-GFP, which results in viral expression of the MHC-I OVA-peptide antigen (SIINFEKL) in infected hepatocytes (Stabenow et al. 2010). As negative control, animals were infected with 2×10^8 PFU Ad-luc-GFP not expressing the OVA antigen. In case functional OVA-specific CTLs would be present in mice following immunization, they would recognize the MHC-I OVA-peptide on the surface of infected hepatocytes and subsequently destroy them. To determine the ability of αDEC-205/OVA induced CTLs to exert antigen-specific effector function following recognition of the MHC-I/SIINFEKL complex on hepatocytes of AdOVA-luc-GFP infected mice, liver influx of $CD8^+$ T lymphocytes (5.1.3.1), liver histology (5.1.3.2), serum ALT levels (5.1.3.3) as well as virus clearance (5.1.3.4) were analyzed and compared with data obtained following BPPcysOVAMPEG and OVA, BPPcysMPEG vaccination.

5.1.3.1 Characterization of the T cell distribution in the liver of immunized mice following hepatic adenovirus infection

To determine, whether αDEC-205/OVA immunization would result in accumulation of antigen-specific $CD8^+$ T cells in the liver following viral infection, which is of crucial importance in terms of HCV-specific immunotherapy, the percentages of $CD8^+$ T lymphocytes in the liver of AdOVA-luc-GFP infected and Ad-luc-GFP infected control mice was assessed by flow cytometry. To gain additional insights in the functionality of $CD4^+$ T cells after liver infection in immunized animals, the percentage of this cellular subset in the liver was determined as well.

As demonstrated in Fig. 21 (left) the percentage of $CD8^+$ T lymphocytes following infection with Ad-luc-GFP was comparably low (~ 8 %) in all groups of immunized mice, whereas the control group treated with αDEC-205 without antigen exhibited the lowest proportion of $CD8^+$ T cells (4.64 %) in the liver. Interestingly, in animals infected with AdOVA-GFP-luc a strong influx of $CD8^+$ T cells was detectable in αDEC-205/OVA immunized mice, where the frequency of $CD8^+$ T cells increased from 8 % in Ad-GFP-luc infected control animals to 16.5 % in mice infected with OVA expressing adenovirus. A doubling in the percentage of $CD8^+$ T cells was also true for BPPcysOVAMPEG immunized mice, where 14.8 % of $CD8^+$ T lymphocytes were detected after infection with AdOVA-GFP-luc compared to 7.68 % after Ad-GFP-luc infection. In contrast to this, considering the frequency of $CD8^+$ T cells after either Ad-GFP-luc control infection or AdOVA-GFP-luc infection the lowest percentage was detectable in αDEC-205 immunized mice (4.64 % and 7.39 %, respectively). Taken together, the observed influx of $CD8^+$ T cells to the virus infected liver was antigen-specific and the consequence of αDEC-205/OVA or BPPcysOVAMPEG mediated expansion of OVA-specific CTLs capable of migrating to the liver in response to viral infections.

Considering the number of $CD4^+$ T cells in livers of mice infected with the control virus, only minor differences within the different groups were detectable (22 - 28 %; Fig. 21, right panel). Interestingly, when comparing AdOVA-GFP-luc and Ad-GFP-luc infected animals, the percentage of $CD4^+$ T cells even decreased in the presence of adenoviral OVA antigen in αDEC-205/OVA (5 %) and BPPcysOVAMPEG (7 %) immunized mice, indicating a shift in the hepatic lymphocyte composition in favor of the $CD8^+$ T cell subset. This observation was not made in the αDEC-205 treated group as already observed before for $CD8^+$ T cells. Unfortunately, isolation of liver lymphocytes from OVA, BPPcysMPEG immunized and AdOVA-GFP-luc infected mice in an amount suitable for flow cytometric analysis failed. For this reason, it was not possible to determine the antigen-specific influx of OVA-specific T cells after viral liver infection in this particular mouse group.

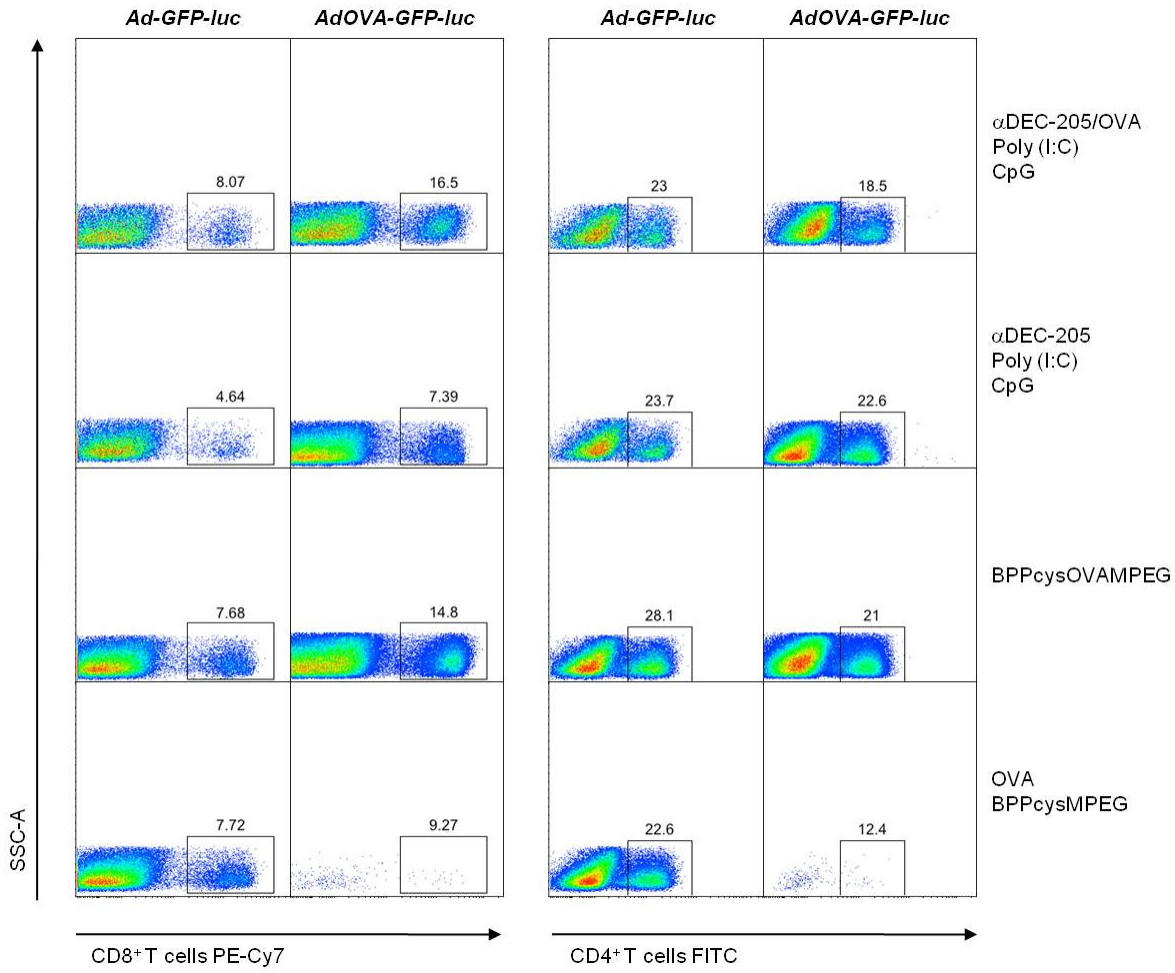

Figure 21. Distribution of CD8$^+$ and CD4$^+$ T cells after Ad-GFP-luc or AdOVA-GFP-luc infection in the liver.

C57BL/6 mice (n = 5) were immunized s.c. in two-week intervals (day 0, 14, 28) with 30 µg αDEC-205/OVA and 30 µg αDEC-205 in addition to the DC maturation stimuli (50 µg Poly (I:C), 50 µg CpG), 10 µg BPPcysOVAMPEG or 7 µg of OVA co-administered with 10 µg BPPcysMPEG. On day 48 after the first injection the animals were infected i.v. with 2 x 10^8 PFU AdOVA-luc-GFP or the control virus Ad-GFP-luc. In order to analyze the percentages of CD8$^+$ and CD4$^+$ T cells in the liver 4 days after infection, liver lymphocytes were isolated and subsequently stained for expression of CD8 and CD4 for FACS analysis. Dot plots represent percentages of CD8$^+$ (left) or CD4$^+$ T cells (right). Abbreviation: plaque forming units (PFU).

5.1.3.2 Histological examination of liver tissue from adenovirus infected mice

To verify antigen-specific CD8$^+$ T cell influx into the liver of αDEC-205/OVA and BPPcysOVAMPEG immunized mice following AdOVA-GFP-luc infection (5.1.3.1) by histological analysis, sections of the upper liver lobe were prepared and stained with hematoxylin and eosin (H&E). Histological examination revealed that neither the liver tissue of αDEC-205/OVA nor BPPcysOVAMPEG immunized mice showed lymphocytic infiltration after control adenovirus infection (Ad-GFP-luc) (Fig. 22A/B). In contrast, infection of αDEC-205/OVA immunized mice with AdOVA-GFP-luc resulted in medium-sized

hepatocellular necrosis accompanied by infiltration of lymphocytes, histiocytes, eosinophiles and neutrophils (arrowhead) as well as lymphocytic and eosinophilic infiltration (arrow) indicating not only the influx of CTLs, but also clearance of AdOVA-GFP-luc infected hepatocytes (Fig. 22C). BPPcysOVAMPEG treated animals showed likewise liver alterations which were, however, not as pronounced as in the αDEC-205/OVA group. In more detail, randomly distributed hepatocellular necrosis (arrowhead) could be observed accompanied by lymphocytic and eosinophilic infiltration (arrow) (Fig. 22D). Regarding liver sections from AdOVA-GFP-luc infected control mice, the αDEC-205 immunized group did not exhibit any abnormalities, whereas livers of OVA, BPPcysMPEG immunized mice showed alteration similar to that found in the BPPcysOVAMPEG immunized group (data not shown).

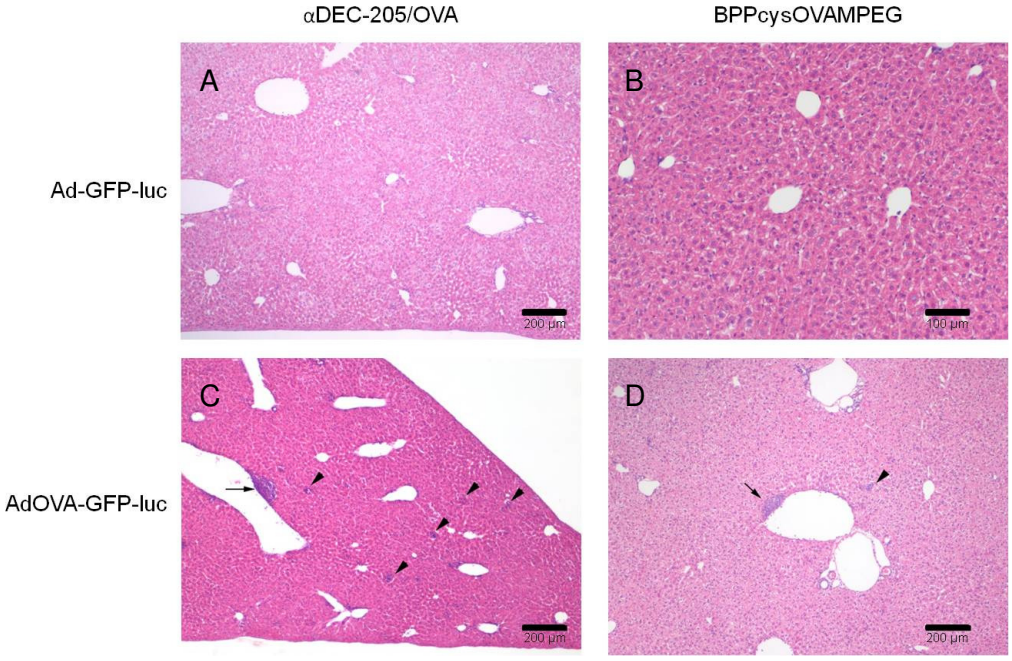

Figure 22. Liver histology of AdOVA-GFP-luc and Ad-GFP-luc infected mice.

C57BL/6 mice (n = 5) were immunized s.c. with either 30 µg αDEC-205/OVA plus 50 µg Poly (I:C) and 50 µg CpG or 10 µg BPPcysOVAMPEG on day 0, 14 and 28. 20 days after the last injection mice were infected i.v. with 2 x 10^8 PFU AdOVA-luc-GFP or the control virus Ad-GFP-luc and sacrificed on day 52. The left upper lobes of the livers were embedded with paraffin, sectioned and stained with H&E. Images show representative overviews (200x or 100x magnification) of liver sections of the indicated experimental groups (arrowhead: hepatocellular necrosis; arrow: lymphocytic and eosinophilic infiltration). Abbreviations: hematoxylin and eosin (H&E); plaque forming units (PFU).

In conclusion, histology confirmed data obtained by FACS analysis demonstrating an antigen-specific influx of lymphocytes into the liver of αDEC-205/OVA and BPPcysOVAMPEG immunized mice after AdOVA-GFP-luc but not Ad-GFP-luc infection.

5.1.3.3 Quantification of serum alanin aminotransferase level as indicator for hepatocyte damage

To further analyze whether $CD8^+$ T cells infiltrating the liver of AdOVA-GFP-luc infected animals would exhibit virus-specific cytotoxic activity, ALT, which is a serum enzyme that is released upon killing of hepatocytes, was measured. To this end, blood was collected from the tail vein of immunized mice before as well as 2 and 3 days after adenovirus infection and ALT level was determined in sera of individual mice.

Infection with Ad-GFP-luc did not result in increased serum ALT levels in all four immunized mouse groups and ALT concentration was found to remain below the critical value of 77 U/l at all days tested (Fig. 23A). This supports the previous observation that the control virus does not activate OVA-specific cytotoxic $CD8^+$ T cells induced in frame of the preceding vaccination (Fig. 21, 22). Unexpectedly, despite liver inflammation following $CD8^+$ T cell influx, serum ALT levels of AdOVA-GFP-luc infected mice did not increase during the indicated time course (day 0, 2 and 3) independent of the immunization approach used (Fig. 23B). A marginal increase of the enzyme indicating hepatocyte killing could be observed in αDEC-205/OVA immunized mice at day 3 after infection (Fig. 23B). This was the reason why in the following experiment the measurement of ALT activity was extended to up to 6 days post infection. Indeed, in both the αDEC-205/OVA and BPPcysOVAMPEG immunized mice the level of the enzyme indicating hepatocyte killing by far exceeded the critical range of 77 U/l, reaching ~ 180 U/l and ~ 210 U/l, respectively (Fig. 23C) proving antigen-specific recognition and $CD8^+$ T cell-mediated killing of AdOVA-GFP-luc infected hepatocytes *in vivo*. Interestingly, ALT concentrations of ~ 90 U/l and thus slightly above the normal level were also detectable in mice immunized with OVA, BPPcysMPEG supporting the data obtained from liver histology showing minimal inflammatory alterations after AdOVA-GFP-luc infection (5.1.3.2).

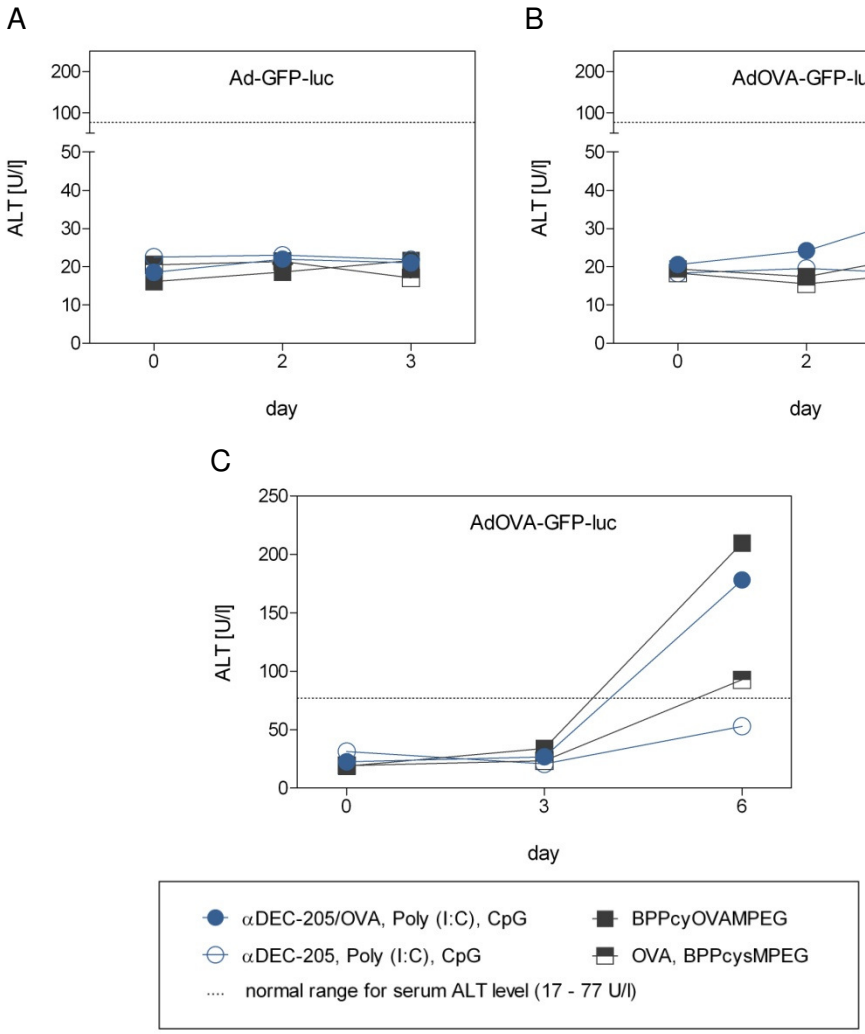

Figure 23. Determination of alanin aminotransferase level in sera of immunized mice following AdOVA-GFP-luc infection.

Mice were immunized s.c. with 30 µg αDEC-205/OVA and 30 µg αDEC-205 in addition to the DC maturation stimuli (50 µg Poly (I:C), 50 µg CpG), 10 µg BPPcysOVAMPEG or 7 µg of OVA co-administered with 10 µg BPPcysMPEG. To quantify hepatocyte damage, serum ALT levels were determined before and after i.v. infection with 2 x 10^8 PFU AdOVA-luc-GFP. Data depicted represent the mean values obtained from n = 5 individual mice and were compared by unpaired, two-tailed *t*-test (ns). A) Mice were immunized s.c. on day 0, 14, 28 followed by challenge with AdOVA-luc-GFP 20 days after the last injection. One representative experiment out of two independent experiments is shown. B) Mice were immunized s.c. on day 0, 14 and infected on day 25 after the first injection. Abbreviations: alanin aminotransferase (ALT); plaque forming units (PFU).

Taken together, as expected from previous experiments, measurement of ALT concentration in sera confirmed hepatocyte damage following AdOVA-GFP-luc infection as an indicator for successful induction of cytotoxic OVA-specific $CD8^+$ T cells in αDEC-205/OVA and BPPcysOVAMPEG immunized mice. On the other hand, immunization with OVA, BPPcysMPEG resulted only in modest hepatocyte killing after viral liver infection, indicating less efficient induction of antiviral CTL responses using this particular vaccination approach.

5.1.3.4 Quantification of adenovirus elimination from the liver of αDEC-205/OVA, OVA, BPPcysMPEG and BPPcysOVAMPEG immunized mice

To finally proof that OVA-specific $CD8^+$ T cells induced during immunization are capable of conferring protective immunity against viral liver infections, elimination of the AdOVA-GFP-luc virus from the liver of mice immunized with αDEC-205/OVA, BPPcysOVAMPEG or OVA, BPPcyMPEG was examined by bioluminescence measurement based on luciferase activity. This was enabled by the fact, that the adenoviral vector AdOVA-GFP-luc carries in addition to the OVA antigen the gene for firefly luciferase (luc), allowing for analyzing luciferase activity (RLU) as an indicator for the relative quantity of AdOVA-GFP-luc infected hepatocytes.

As depicted in Fig. 24, hepatocytes infected with the OVA-expressing adenovirus were efficiently killed in αDEC-205/OVA and BPPcysOVAMPEG immunized mice. Virus elimination was significantly stronger in αDEC-205/OVA *(* P = 0.0339)* and BPPcysOVAMPEG *(** P = 0.0040)* treated animals when compared to the αDEC-205 immunized control group. Of note, no virus clearance was observed in mice that received OVA, BPPcysMPEG treatment, further underlining inefficient induction of CTLs by this approach.

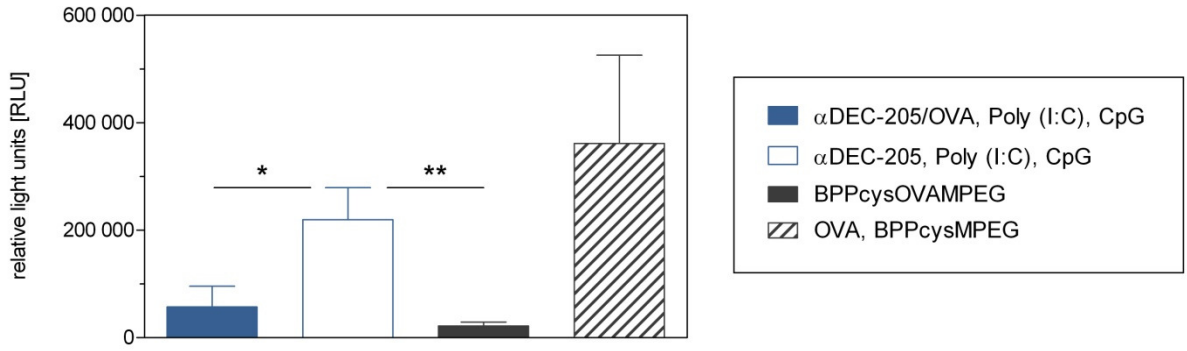

Figure 24. OVA-specific killing of hepatocyte in αDEC-205/OVA immunized mice after AdOVA-GFP-luc infection.

In order to test whether antigen expression in hepatocytes leads to OVA-specific killing in the liver of s.c. immunized mice (day 0, 14, 28), luciferase activity was determined induced by 2 x 10^8 PFU recombinant adenovirus-OVA (AdOVA-luc-GFP) infection applied i.v. (day 48). Mice were s.c. immunized mice on day 0, 14, 28 with 30 µg αDEC-205/OVA and 30 µg αDEC-205 in addition to the maturation stimuli (50 µg Poly (I:C), 50 µg CpG), 10 µg BPPcysOVAMPEG or 7 µg of OVA co-administered with 10 µg BPPcysMPEG. For quantification of luciferase activity mice were sacrificed on day 52 after the first immunization and liver homogenates (2 samples for one mouse) were analyzed at independent time points in a luminometer. One representative experiment of two is displayed and results are expressed in (RLU) as means ± SEM (n = 5). Unpaired, two-tailed *t*-test was used for statistical analysis *(** P = 0.0040, * P = 0.0339)*. Abbreviations: relative light units (RLU); plaque forming units (PFU); standard error of the mean (SEM).

In summary, data obtained by bioluminescence measurement clearly demonstrated that both αDEC-205/OVA as well as and BPPcysOVAMPEG immunization efficiently induces CTLs that are capable of recognizing and killing virus infected liver cells. This is well in line with data obtained by FACS analysis which revealed antigen-specific influx of $CD8^+$ T lymphocytes into the virus infected liver cells (Fig. 21) In addition, histological examination revealed hepatocellular necrosis and infiltration of lymphocytes and eosinophils in the liver of αDEC-205/OVA and BPPcysOVAMPEG immunized mice following AdOVA-GFP-luc infection (Fig. 22), which correlated well with increased serum ALT levels as reliable marker for CTL-mediated liver damage (Fig. 23).

To conclude data obtained in the first part of this thesis, targeting OVA protein to DEC-205^+ DCs induces the full repertoire of adaptive immune responses being crucial for the development of a potent antiviral vaccine. αDEC-205/OVA immunization lead to a Th1-dominated effector T cell response indicated by the pronounced IFNγ release (Fig. 19) and a fast and vigorous antibody response (Fig. 20). Of note, antigen-specific CTLs were effectively primed (Fig. 18) and these cells exert antigen-specific effector function as they were found to clear virus infected hepatocytes. Since DEC-205-mediated antigen delivery to DCs was found to be in many ways superior to the BPPcysMPEG based approach, this strategy was chosen for extending vaccination studies to HCV-related antigens in frame of the second part of this thesis

5.2 Part II

Generation of αDEC-205/HCV antigen conjugates and analysis of adaptive immunity induced following DEC-205-mediated delivery of HCV antigens to dendritic cells

To study the adequacy of DEC-205-mediated *in vivo* targeting of antigen to DCs for the intended use as an immunotherapeutic tool against HCV infection, conjugates consisting of αDEC-205 and the HCV proteins NS3 and Core were generated and tested. These particular HCV proteins were selected because they are highly conserved and moreover, have been identified as attractive candidate antigens suitable for the use as HCV vaccine, since control of HCV infection is usually associated with multi-specific $CD4^+$ and $CD8^+$ T cell responses to different structural and non-structural HCV proteins, including Core and NS3 (Bukh et al. 1994; Yasui et al. 1998; Yu et al. 2004; Leroux-Roels 2005; Neuman-Haefelin et al. 2005; Bowen & Walker 2005; Rehermann & Nascimbeni 2005; Cao et al. 2011). Recombinant expression and subsequent purification of the HCV proteins NS3 (aa 1027-1218) and Core (aa 2-191) were established on the basis of protocols published by Vishnuvardhan et al. and Mihailova et al., respectively, followed by chemical conjugation of the antigens to αDEC-205 (Vishnuvardhan et al. 1997, Mihailova et al. 2006). Finally, the impact of the chemical conjugation on the αDEC-205 binding capacity was analyzed and immunization trials with the αDEC-205/NS3 as well as αDEC-205/Core conjugates were performed.

5.2.1 HCV NS3

5.2.1.1 Optimization of the HCV NS3 protein purification

In order to obtain high level expression of the HCV NS3 protein, *E. coli* BL21 (DE3) expressing the codon-optimized cDNA sequence of NS3 aa 1027-1218 were cultured in 1 liter TB-medium to an OD_{600} of 0.5 followed by induction of protein expression by the addition of IPTG. Since the recombinant NS3 protein was not secreted but remained intracellular, bacteria were harvested by centrifugation and, in contrast to the published protocol, re-suspended in lysis buffer supplemented with lysozyme followed by mechanical disintegration using a French press instead of sonification. After purification of the NS3 protein as described in Materials and Methods, purity of the eluates was analyzed by SDS-PAGE and Coomassie blue staining. As depicted in Fig. 25 purifying the protein under native conditions using a step-wise pH gradient resulted in successful isolation of highly pure NS3 protein exhibiting 21 kDa in size. Unfortunately, increasing the concentration of the NS3 protein as well as increasing the pH during the purification procedure resulted in precipitation of the

protein. However, addition of 1.5 M urea to the NS3 elution buffer and fast handling could partially prevent this.

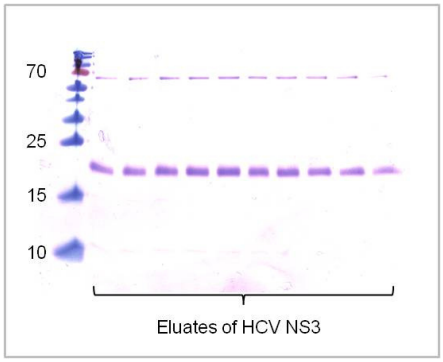

Figure 25. Purification of the HCV NS3 protein (aa 1027-1218).

Recombinant NS3 protein expression was carried out in BL21 (DE3) expression-ready-clone HCV NS3 after induction with 1 mM IPTG. Purification of the NS3 protein was performed under native conditions utilizing a stepwise decrease of the pH in the buffer used for washing and elution. Eluate samples were loaded on a 15 % polyacrylamide gel und subjected to SDS-PAGE and Coomassie blue staining. Recombinant NS3 was detectable as a protein band of 21 kDa in size. Abbreviations: Isopropyl-,-D-thiogalactopyranosid (IPTG); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

To obtain higher amounts of the NS3 antigen, which was needed for chemical conjugation to the DEC-205 antibody (at least 0.5 mg), it was necessary to either scale up bacterial growth culture (i.e. 6 liter culture) or to pool different NS3 protein batches obtained by independent purifications.

5.2.1.2 Conjugation of the HCV NS3 protein to αDEC-205

After having optimized the HCV NS3 protein purification procedure, it was next studied whether this protein could be conjugated to the DEC-205 antibody on the basis of the protocol established for the model antigen OVA (5.1.1). The conjugation efficiency of HCV NS3 (aa 1027-1218) to αDEC-205 was limited by two fundamental points: First, it was necessary to find a conjugation buffer suitable for the sulfo-SMCC-based chemical crosslinking reaction and at the same time preventing precipitation of the NS3 protein. Second, due to the extensive purification procedure, the NS3 amount available for the chemical conjugation was (in contrast to OVA) quite limited.

Despite considerable experimental effort, the challenge to chemically conjugate purified NS3 (aa 1027-1218) to αDEC-205 turned out to be extremely complicated. Most notably, the necessity to increase the pH from acidic to neutral in order to guarantee optimal functionality of the chemical crosslinker resulted in precipitation of the NS3 protein. Consequently, only very small amounts of NS3 protein were available which, however, did not allow the efficient conjugation to αDEC-205 (data not shown). This was the reason why self-purified NS3 (aa

1027-1218) was substituted by recombinant NS3 (genotype 1b, aa 1192-1459, GST-tagged) obtained from a company, which was used for the following experiments. Indeed, NS3 (aa 1192-1459) conjugation to the DEC-205 antibody was successful as demonstrated by NS3-specific western blot analysis (Fig. 26). Here, the αDEC-205/NS3 conjugate is represented by the ~205 kDa protein band. As expected, unconjugated NS3 protein was detectable as a fragment 55.4 kDa in size (29.4 kDa NS3 + 26 kDa GST) as visualized by staining with a primary antibody specific for HCV-NS3 [20-8] followed by secondary staining with an α-mouse HRPO antibody.

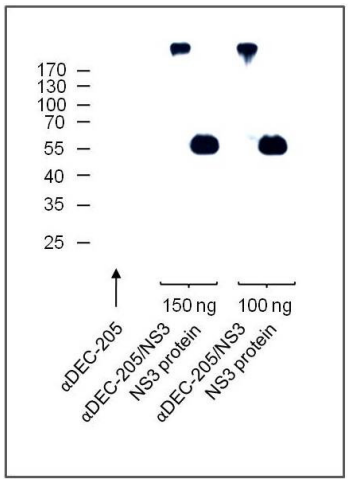

Figure 26. Conjugation of HCV NS3 (aa 1192-1459) to αDEC-205.

The αDEC-205/NS3 conjugate was generated on the basis of the protocol established for OVA conjugation using the sulfo-SMCC crosslinker and TCEP. NS3 protein, αDEC-205 and αDEC-205/NS3 conjugate samples were subjected to 12 % SDS-PAGE followed by Western Blot analysis. Successful conjugation was verified by staining with mouse α-HCV NS3 [20-8] followed by a secondary incubation with donkey α-mouse HRPO. Conjugation of αDEC-205 to the NS3 protein resulted in a protein ~205 kDa in size. The GST-tagged NS3 alone exhibits a size of 55.4 kDa. Abbreviations: horseradish peroxidase (HRPO); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC); tris(2-carboxyethyl)phosphine (TCEP).

Conjugation efficiency was found to be very high, since no free NS3 was detectable after chemical crosslinking (Fig. 26). However, since subsequent immunization of mice with the αDEC-205/NS3 conjugate required high concentrations of the conjugate in volume small enough for single injection in the hind footpads, liquid volume was reduced using Vivaspin 20 columns in order to increase the antigen concentration in the samples. However, as a consequence of losing considerable amounts of the protein or the conjugate during the workflow (purification, concentration, conjugation, concentration), it was not possible to yield a αDEC-205/NS3 conjugate concentration of 30 µg/50 µl, which was proven before in frame of the αDEC-205/OVA immunization trials to induce robust antiviral immunity.

5.2.1.3 Characterization of the capacity of the αDEC-205/NS3 conjugate to bind to the endocytosis receptor DEC-205 on dendritic cells

Since western blot analyses revealed efficient conjugation of NS3 (aa 1192-1459) to αDEC-205 (5.2.1.2), it was next studied, whether the conjugate maintains its capacity to bind to DEC-205 expressed on the surface of dendritic cells. In order to address this question FACS analyses were performed.

BMDCs were prepared as described in Materials and Methods and incubated with 10 µg/ml of the αDEC-205/NS3 conjugate, αDEC-205 or medium, which served as control. Subsequently, the cells were stained with either PE-labeled goat α-rat to detect the DEC-205 antibody or with a primary antibody directed against HCV NS3 [20-8] followed by staining with the secondary antibody specific for mouse IgG_1 (PE-labeled) to visualize the conjugated NS3 antigen. As depicted in Fig. 27A, 5.38 % of αDEC-205 treated BMDCs stained positive for rat IgG indicating binding of the antibody to DEC-205 on the surface of BMDC (Fig. 27A). In contrast, almost no rat IgG-positive cells (0.06 %) were detectable in the αDEC-205/NS3 treated group and the percentage of αDEC-205/NS3 treated BMDCs positive for NS3 was as well very low (0.40 %; Fig. 27B), This suggested either impaired binding of the αDEC-205/NS3 conjugate to the DEC-205 molecule on the surface of DCs or impaired antibody binding to the αDEC-205/NS3 conjugate due to conformational changes as a consequence of chemical crosslinking, therefore making it impossible to detect both, the DEC-205 antibody as well as the NS3 protein by FACS analysis.

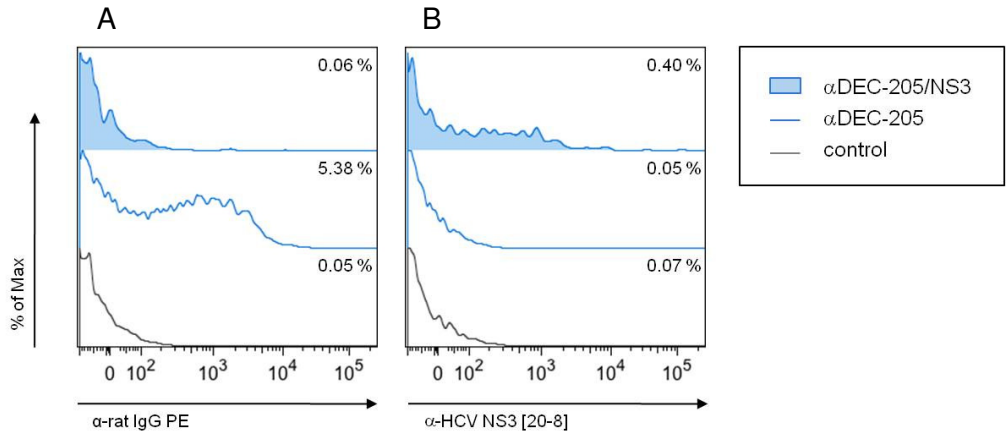

Figure 27. Binding analysis of αDEC-205/NS3 to bone-marrow derived cells *in vitro*.

FACS analysis was performed to examine the binding capacity of the αDEC-205/NS3 conjugate to BMDCs *in vitro*. Cells were incubated with 10 µg/ml αDEC-205/NS3, αDEC-205 or medium, which served as control (1 hour at 4°C) followed by staining with APC-labeled αCD11c. In order to detect αDEC-205/NS3 bound to BMDCs, the cells were also stained with either PE-labeled goat α-rat (A) or α-HCV NS3 [20-8] followed by secondary staining with α-mouse IgG1 PE (B). Histograms represent $CD11c^+$ cells stained positive for PE fluorescence. One representative experiment out of 3 independent is displayed. Abbreviations: fluorescence-activated cell sorting (FACS); bone-marrow derived cells (BMDCs).

To further investigate whether or not chemical crosslinking of NS3 to αDEC-205 would interfere with the binding capacity of the conjugate, immunization studies were performed.

5.2.1.4 Immunization with αDEC-205/NS3

In order to study, whether the αDEC-205/NS3 conjugate would be capable of binding to DEC-205 on DCs and whether immunization with the conjugate would induce NS3- and therefore HCV-specific cellular and humoral immune responses comparable to those observed after αDEC-205/OVA injection, vaccination trials were performed. Due to technical limitation (see above) the maximal amount of 5 µg of the αDEC-205/NS3 conjugate could be injected per mouse at every day of immunization instead of 30 µg which has been used before for αDEC-205/OVA immunization.

- ***Cellular immune responses***

In a first experiment, mice were immunized s.c. intra footpad with 5 µg αDEC-205/NS3 or NS3 in the presence of 25 µg αCD40 and 50 µg Poly (I:C) on day 0 followed by a boost 14 days later. Injection of 5 µg αDEC-205/NS3 without maturation stimuli served as control. At day 28 after the first injection spleens and popliteal lymph nodes were isolated, single cell suspensions were prepared and re-stimulated with the NS3 protein *in vitro*. Proliferative capacity of splenocytes and lymph node cells was analyzed using ^{3}[H]-thymidine incorporation assays.

As indicated in Fig. 28, the NS3-specific T cell proliferation induced in the αDEC-205/NS3, Poly (I:C), αCD40 immunized group was significantly higher in the spleen *(** p = 0.0012)* and popliteal lymph nodes *(** p = 0.0019)* when compared to the group immunized with αDEC-205/NS3 without maturation stimuli. However, no significant differences in T cell proliferation were observed comparing the αDEC-205/NS3 and NS3 (both with Poly (I:C) and αCD40) immunized groups. Thus, twice immunizations with as few as 5 µg of soluble NS3 protein or NS3 conjugated to αDEC-205 induced systemic T cell responses. Since an absolute amount of 5 µg of NS3 protein and the αDEC-205/NS3 conjugate was used for immunization, the absolute amount of antigen applied to the mice was about 50-fold higher in case of NS3 injection when compared to αDEC-205/NS3 immunization as estimated by western blot analysis (data not shown). Therefore, when considering these substantial differences in the antigen dose used for the immunizations, targeting the HCV protein to DEC-205 on the surface of DCs was found to be superior compared to immunization with soluble NS3 in inducing cellular immunity.

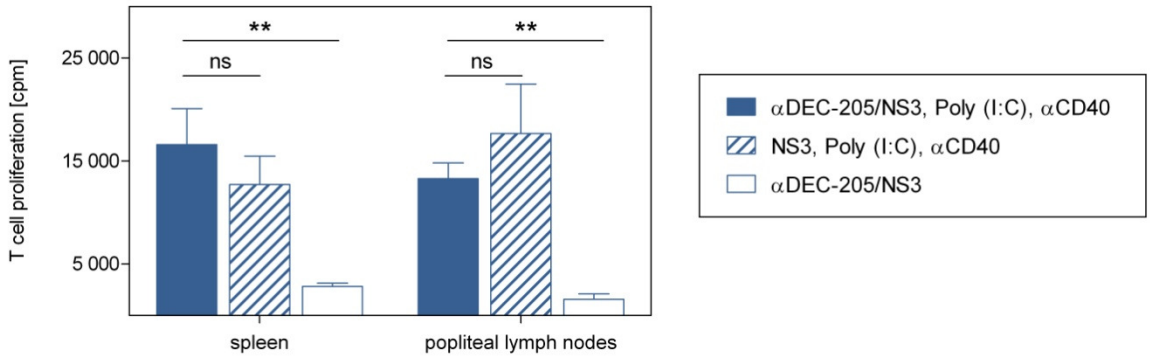

Figure 28. Cellular immune responses after αDEC-205/NS3 immunization.

Balb/c (n = 3) were immunized s.c. in the hind footpads with 5 µg αDEC-205/NS3 or NS3 in the presence of 25 µg αCD40 and 50 µg Poly (I:C) on day 0 followed by a boost 14 days later. Injection of 5 µg αDEC-205/NS3 without maturation stimuli served as control. Mice were sacrificed on day 28 after the first injection. *In vitro* T cell proliferation was evaluated by re-stimulating single cell suspension of spleen or popliteal lymph nodes with 1 µg/ml NS3 protein (aa 1192-1459) in triplicates using ^{3}[H]-thymidine incorporation assay. Results were expressed as the mean ± SEM of [^{3}H] thymidine uptake in cpm. Statistical analysis has been performed by unpaired, two-tailed *t*-test *(** $p < 0.002$)*. One independent experiment is displayed. Abbreviations: counts per minute (cpm); standard error of the mean (SEM).

- ***Humoral immune responses***

To test, whether αDEC-205/NS3 immunization would also induce humoral immune responses, total IgG (Fig. 29A) as well as different IgG subtypes (Fig. 29B) were quantified. To this end, mice were immunized twice on day 0 and 14 (Fig. 29A) or over a longer period (8 weeks in total; every second week) (Fig. 29B), followed by preparation of serum samples and ELISA. Similar amounts of NS3-specific IgG were detectable in sera of all 3 mouse groups tested, independent of the vaccination approach used (Fig. 29A). To further define the nature of the antibody response induced after immunization with the αDEC-205/NS3 conjugate or soluble NS3 protein, the IgG subclasses were analyzed by ELISA. In both immunized mouse groups, predominantly IgG_1 and IgG_{2a} were induced, with IgG_{2a} showing the highest serum concentration (Fig. 29B). There were, however, no significant differences in IgG subclasses detectable between the αDEC-205/NS3 and NS3 protein groups.

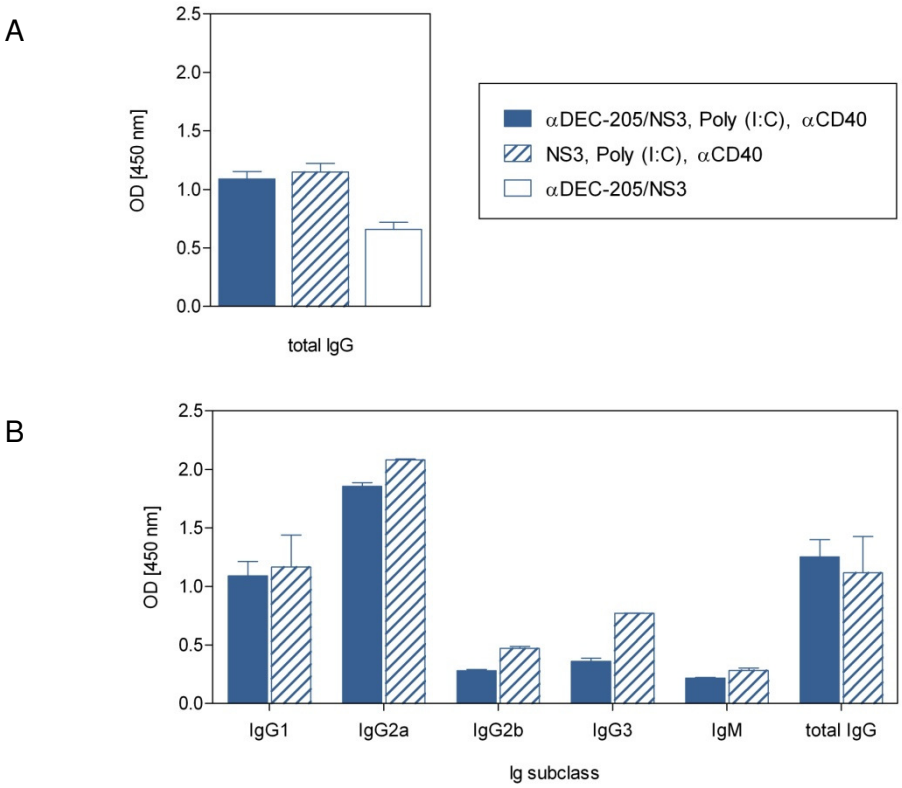

Figure 29. Humoral immune responses after αDEC-205/NS3 immunization.

Balb/c (n = 3) were immunized s.c. in the hind footpad with 5 µg αDEC-205/NS3 or NS3 in the presence of 25 µg αCD40 and 50 µg Poly (I:C) on day 0 followed by one (day 14) (A) or three boosts (day 14, 28, 42) (B) and were sacrificed 14 days after the last vaccination. Injection of 5 µg αDEC-205/NS3 without maturation stimuli served as control. A) Blood was collected from mice 28 days after first immunization. ELISA plates were coated with 2 ng/µl NS3 (aa 1192-1459) and total IgG in sera diluted 1:500 was determined. Bars represent mean values (n = 3) of the absorbance [OD_{450}] in duplicates. B) Sera from long-term immunized mice (diluted 1:500) were tested for NS3-specific IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3) and IgM. Results are expressed as mean values (n = 3, duplicates). Abbreviation: ELISA enzyme-linked immunosorbent assay

In summary, initial immunization trials using the αDEC-205/NS3 conjugate revealed induction of systemic and antigen-specific cellular and humoral immune responses. Considering the absolute amount of NS3 antigen used for the immunization, with considerably lower absolute amounts of NS3 included in the αDEC-205/NS3 conjugate compared to immunization using soluble NS3, it can be concluded that *in vivo* targeting of antigen to DCs via the DEC-205 antibody is superior in inducing specific immunity compared to immunization with soluble antigen. However, despite extensive effort, it was not possible to reproduce the data from the first immunization trial, i.e. subsequently performed experiments using a newly produced αDEC-205/NS3 batch did not result in the induction of NS3-specific T cell responses. This may be attributed to variations in conjugation efficiency, limitation in antigen dose and/or impaired binding capacity of the conjugate, which all represent parameters that may vary between each individual crosslinking reaction and thereby explaining different outcomes in independent immunization experiments.

5.2.2 HCV Core

5.2.2.1 Optimization of the HCV Core protein purification

The second HCV antigen selected as candidate for vaccination studies was the HCV Core protein. Core purification was performed under denaturing conditions. Only the washing step included a pH gradient (pH 6.0, pH 5.9, pH 5.5) and elution of the Core protein from the column was carried out at pH 4.5. This purification protocol resulted in high amounts of Core in the eluate, which was highly pure as indicated in Fig. 30. To facilitate subsequent conjugation to the DEC-205 antibody, the pH of the buffer was increased to 7.0 and at the same time the urea concentration was decreased from 8 M to 1.5 M using 10.000 MWCO Vivaspin 20 columns. In contrast to the NS3 protein, increasing the Core concentration and modifying the buffer conditions did not result in precipitation of the protein.

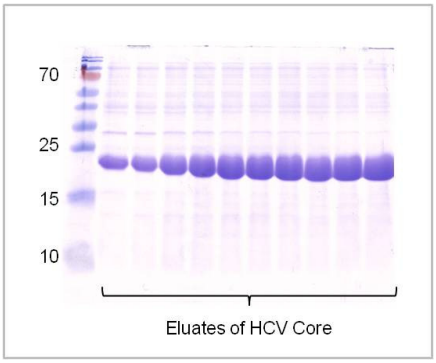

Figure 30. Purification of the HCV Core protein (aa 2-191).

Protein expression was carried out in expression-ready-clone HCV Core hosted by the bacteria BL21 (DE3) and induced with 1 mM IPTG. Purification of the Core protein was performed under native conditions utilizing a stepwise decrease of the pH in the buffer used for washing and elution. Elution samples of HCV Core were loaded on 15 %-SDS-PAGE gel and fragments of Core (20.8 kDa) were visualized by Coomassie staining. Abbreviations: Isopropyl-,-D-thiogalactopyranosid (IPTG); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

5.2.2.2 Conjugation of the HCV Core to αDEC-205

Having established the experimental conditions for HCV Core (aa 2-191) purification, it was next studied, whether this protein could be chemically conjugated to αDEC-205 using the protocol established for OVA (5.1.1). As already mentioned before for the NS3 protein, successful conjugation was dependent on the selection of optimal buffer conditions supporting chemical reactivity of the crosslinker sulfo-SMCC, while at the same time ensuring high stability and solubility of the HCV Core protein (5.2.1.2). Moreover, due to the complex purification procedure, the amount of protein available for chemical cross-linking was limited. In contrast to the in-house produced NS3 protein the HCV Core turned out to be more stable in its respective buffer (1.5 M urea; 10 mM Tris-HCl, 100 mM NaH_2PO_4) which

was compatible with the chemical crosslinking reaction enabling the generation of αDEC-205/Core using the self-made protein. Of note, the conjugation of recombinant Core (aa 2-119; multiple GST-tags) purchased by a company to αDEC-205 was also tested in order to circumvent the limited access to HCV Core due to the extensive purification procedure, but this approach did not succeed (data not shown). Thus, purified and TCEP-activated Core was crosslinked to sulfo-SMCC activated αDEC-205, followed by analysis of the conjugation efficiency by Western Blot analysis using α-HCV Core [C7-50] as primary and donkey α-mouse-HRPO as secondary antibody. Detection of a protein band >170 kDa in size indicated successful conjugation of Core to the DEC-205 antibody. As expected, unbound Core exhibited a size of ~20.8 kDa. As depicted in Fig. 31, conjugation efficiency was very high and no free Core protein was detectable in the αDEC-205/Core sample. Thus, whereas no further purification step was needed, it was necessary to reduce the overall volume of the αDEC-205/Core sample in order to increase the antigen concentration for the subsequent immunization experiments. However, as for the αDEC-205/NS3 conjugate it was not possible to yield a αDEC-205/Core concentration of 30 µg/50 µl, which was proven before in frame of the αDEC-205/OVA immunization trials to induce robust antiviral immunity.

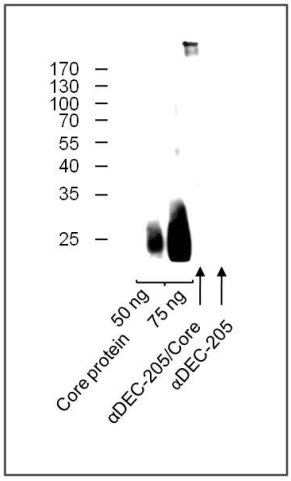

Figure 31. Conjugation of HCV Core (aa 2-191) to αDEC-205.

The αDEC-205/Core conjugate was generated on the basis of the protocol established for OVA conjugation using the crosslinker sulfo-SMCC and TCEP. Protein and conjugate samples were subjected to 12 % SDS-PAGE and subsequent Western Blot analysis. Successful conjugation was verified by staining with mouse α-HCV Core [C7-50] followed by a secondary staining step using donkey α-mouse HRPO. Conjugation of αDEC-205 to Core resulted in protein bands of >170 kDa in size. In contrast, a fragment of 20.8 kDa in size was detectable for the soluble Core. Abbreviations: horseradish peroxidase (HRPO); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC); tris(2-carboxyethyl)phosphine (TCEP).

5.2.2.3 Assessment of the conjugation efficiency by ELISA

In addition to Western Blot analysis (5.2.2.2), an ELISA-based approach was used to demonstrate successful conjugation of HCV Core to αDEC-205.

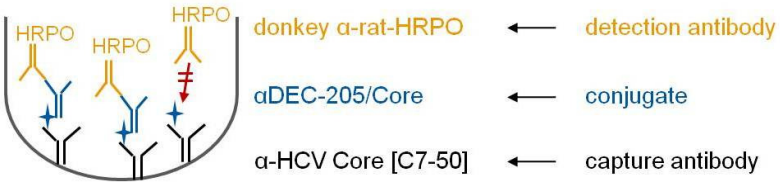

Figure 32. Strategy to prove chemical conjugation of HCV Core to αDEC-205.

The capture antibody α-HCV Core [C7-50] recognizes both the conjugated HCV protein and the soluble Core in the sample of αDEC-205/Core. In contrast, the HRPO-labeled donkey α-rat IgG detecting the rat-derived αDEC-205 will only bind to the conjugate and not to the soluble Core protein providing a basis to demonstrate successful conjugation. Abbreviation: horseradish peroxidase (HRPO).

As schematically shown in Fig. 32 ELISA plates were coated with the α-HCV Core [C7-50] antibody and subsequently incubated with the αDEC-205/Core conjugate. In case Core would be conjugated to αDEC-205 the α-HCV Core antibody would specifically bind to it. Thus, in a second step, incubation with the α-rat-HRPO antibody would allow binding to the rat-derived DEC-205 antibody, which could be visualized by an enzymatic reaction detected by measuring OD_{450}. As summarized in Table 9, this was indeed the case. Incubation with the αDEC-205/Core conjugate resulted in an OD_{450} = 0.629 compared to OD_{450} = 0.198 following incubation with αDEC-205 and OD_{450} = 0.187 for incubation of the α-HCV Core [C7-50] coated plates with soluble Core. Thus, the successful chemical conjugation of αDEC-205 to Core could also be demonstrated by ELISA.

Coated with	primary antibody	OD [450 nm]
mouse α-HCV Core [C7-50]	**αDEC-205/Core**	**0.629**
	αDEC-205	0.198
	Core	0.187
PBS	αDEC-205/Core	0.074
	αDEC-205	0.076
	Core	0.044

Table 9. Analysis of the αDEC-205/Core conjugate by ELISA.

ELISA plates were coated with mouse α-HCV Core [C7-50] or PBS. Subsequently, the αDEC-205/Core conjugate, αDEC-205 or Core was added to the plate. After washing donkey α-rat IgG was added and the successful conjugation was visualized using the TMB Liquid substrate system combined with 2.5 M H_2SO_4-solution and detected by OD_{450} measurement.

5.2.2.4 Characterization of the capacity of the αDEC-205/Core conjugate to bind to the endocytosis receptor DEC-205 on dendritic cells

Since both Western Blot analysis and ELISA revealed efficient conjugation of the Core protein to αDEC-205 (5.2.2.2/3), in a next step flow cytometry and immunofluorescence microscopy analyses were performed in order to rule out that the chemical crosslinking reaction may negatively influence the DEC-205 antibody binding capacity.

- ***FACS analysis***

BMDCs were prepared as described in Materials and Methods and incubated with 10 µg/ml of the αDEC-205/Core conjugate, αDEC-205 alone or medium, which served as control. Subsequently, the cells were stained with either PE-labeled goat α-rat to detect the DEC-205 antibody or with a primary antibody directed against HCV Core [C7-50] followed by staining with the secondary antibody specific for mouse IgG_1 (PE-labeled) to visualize the conjugated Core antigen. Of note, the αDEC-205/Core conjugate efficiently bound to DEC-205 on BMDCs as indicated by positive staining with both α-rat IgG (27.9 %, Fig. 33A) and α-HCV Core (10.9 %, Fig. 33B). As already observed before (Fig. 27), only 5.38 % of BMDCs incubated with αDEC-205 BMDCs stained positive for rat IgG (Fig. 33A) and, as expected, were negative for the HCV Core protein (Fig. 33B). Together, this clearly demonstrates that crosslinking Core to the DEC-205 antibody does not interfere with its capacity to bind to its target molecule on the surface of BMDC.

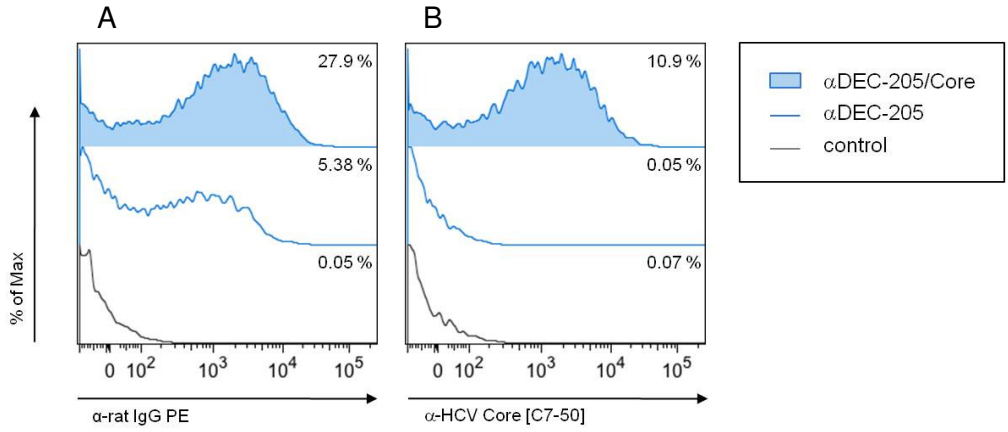

Figure 33. Binding analysis of αDEC-205/Core to bone-marrow-derived cells *in vitro*.

FACS analysis was performed to examine the binding capacity of αDEC-205/Core to its target molecule on the surface of BMDCs *in vitro*. Cells were incubated with 10 µg/ml αDEC-205/Core, αDEC-205 or medium, which served as control (1 hour at 4°C) followed by staining with APC-labeled αCD11c. In order to detect αDEC-205/Core bound to the surface of BMDCs, the cells were additionally stained with either PE-labeled goat α-rat (A) or α-HCV Core [C7-50] followed by secondary α-mouse IgG_1 PE staining (B). Histograms represent $CD11c^+$ cells stained positive for PE fluorescence. One representative experiment out of 3 independent experiments is displayed. Abbreviation: bone-marrow derived cells (BMDCs).

Next to the antibody binding analysis under native conditions using BMDCs, binding of the αDEC-205/Core conjugate was tested on splenocytes. To this end, splenocytes were incubated with either the conjugate (αDEC-205/Core), the antibody alone (αDEC-205) or soluble Core protein, control cells were left untreated. Subsequently, splenocytes were fixed with PFA and stained for the DEC-205 antibody using a FITC-labeled goat α-rat antibody and in addition for CD11c expression to identify DCs. FACS analysis revealed that 27.9 % of the αDEC-205/Core and 14 % of αDEC-205 treated splenic DCs stained positive with the FITC-labeled goat α-rat antibody (Fig. 34), corroborating data obtained before showing that chemical crosslinking of Core to αDEC-205 does not negatively influence the capacity of the antibody to bind to DEC-205 on DCs.

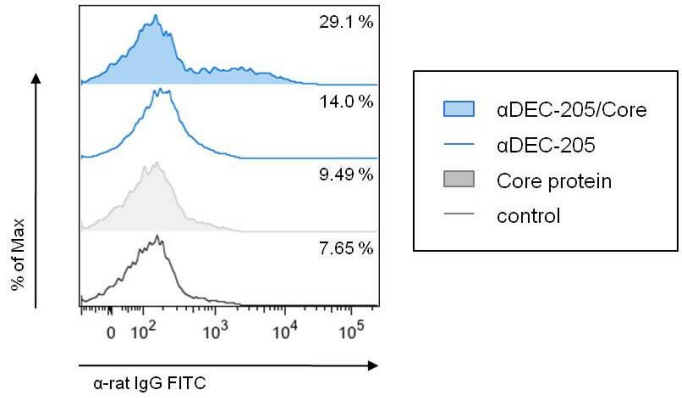

Figure 34. Binding analysis of αDEC-205/Core to CD11c$^+$ splenocytes *in vitro*.

FACS analysis was performed to examine the ability of the conjugates to bind to DEC-205 expressed by isolated splenocytes *in vitro*. For this, cells were incubated with 10 µg/ml αDEC-205/Core, αDEC-205 or medium, which served as control (1 hour at 4°C). Subsequently, the cells were fixed with PFA and stained with FITC-labeled goat α-rat. To enable gating on CD11c$^+$-positive cells, splenocytes were also stained with APC-labeled αCD11c. Gated CD11c$^+$-positive single lymphocytes were displayed in a histogram and percentages of cells stained positive for FITC fluorescence are shown. One independent experiment is displayed. Abbreviations: fluorescence-activated cell sorting (FACS); paraformaldehyde (PFA).

- ***Immunofluorescence microscopy***

In order to further proof that the αDEC-205/Core conjugate binds to DCs, MHC II^+/$CD11c^+$ BMDCs were sorted and subsequently incubated with αDEC-205/Core, αDEC-205 or Core. Next, the cells were fixed and stained with α-rat IgG Alexa594 and α-HCV Core [C7-50] followed by another staining step using α-mouse IgG Alexa 488. Finally, the cells were examined by Immunofluorescence microscopy. As shown in Fig. 35A bright dots indicate proper binding of the αDEC-205/Core conjugate to DCs. Moreover, immunofluorescence microscopy of the same cells also revealed presence of the Core protein as indicated by the bright dots in Fig. 35B. An overlay of the two pictures confirmed co-localization of αDEC-205 (red) and Core (green), which together results in an orange fluorescence signal (Fig. 35C), indicating efficient binding of the conjugate to MHC II^+/$CD11c^+$ DCs. As expected, microscopic analysis of BMDCs incubated with soluble Core before antibody staining revealed no positive staining for the Core protein emphasizing that conjugation to αDEC-205 is required for targeting Core to DCs (data not shown).

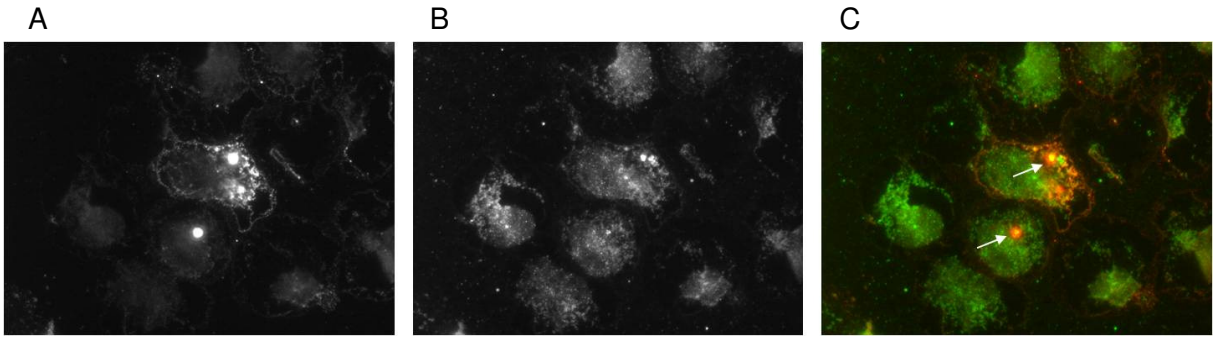

Figure 35. Binding analysis of αDEC-205/Core by immunofluorescence microscopy.

Generated BMDCs from naïve Balb/c mice were sorted for their MHC II^+- and $CD11c^+$-expression and were incubated with 10 µg/ml αDEC-205/Core. Following washing, cell-bound αDEC-205/Core was simultaneously stained with antibodies specific for αDEC-205 (α-rat Alexa 594) (A) and for Core (α-HCV Core [C7-50], α-mouse IgG Alexa 488) (B) for 30 minutes at 4°C. After fixing the coverslips on microscopy slides the binding ability to BMDCs of αDEC-205/Core was visualized by immunofluorescence microscopy. C) An overlay of both stainings (double positive = orange) suggested binding ability of αDEC-205/Core to BMDCs. One representative out of two independent experiments is displayed. Abbreviation: bone-marrow derived cells (BMDCs).

In summary, both FACS analyses and immunofluorescence microscopy revealed that chemical crosslinking of Core to αDEC-205 did not impair the binding capacity of the conjugate and that αDEC-205/Core is capable of specifically targeting the Core antigen to the endocytosis receptor DEC-205 on the surface of DCs.

5.2.2.5 Immunization with αDEC-205/Core

In order to study, whether immunization with the αDEC-205/Core conjugate would lead to induction of Core- and therefore HCV-specific cellular and humoral immune responses vaccination studies were performed in mice. As already mentioned for the αDEC-205/NS3 conjugate only 5 µg of the αDEC-205/Core conjugate instead of 30 µg proven before in frame of the αDEC-205/OVA immunization trials to induce robust antiviral immunity could be used for vaccination due to technical limitations in protein purification and conjugation (5.2.1.4).

- ***Cellular immune responses***

To study, whether *in vivo* targeting of the Core antigen to DCs by utilizing the αDEC-205/Core conjugate would efficiently induce humoral and cellular immune responses, mice were immunized twice each with 5 µg of the αDEC-205/Core conjugate or αDEC-205 as negative control, respectively on day 0 and 7. A further group was immunized with soluble Core protein. In order to inject an antigen dose comparable to that used for the αDEC-205/Core treated group, Core concentration within the conjugate was estimated semi-quantitatively by Western Blot analysis and ELISA (data not shown) and based on the results obtained the absolute amount of soluble Core used for immunization was adjusted to 1.25 µg. All mouse groups also received 50 µg Poly (I:C) and 50 µg CpG to induce maturation of DCs. 14 days after the first immunization, mice were sacrificed, single-cell suspension were generated from the spleens, followed by MACS enrichment of T cells and *in vitro* re-stimulation with Core (aa 2-119) pulsed BMDCs. Proliferative capacity of T cells in these cultures was assessed by ^{3}[H]-thymidine incorporation assay. Unexpectedly, no Core-specific T cell proliferation was detectable in the spleen of all groups tested. In contrast, Core-specific T cell proliferation was observed in lymph nodes draining the site of antigen injection. Here, T cells from αDEC-205/Core immunized mice responded significantly better to Core re-stimulation compared to T cells isolated from the popliteal lymph nodes of Core *(** $p = 0.0034$)* or αDEC-205 *(** $p = 0.0070$)* immunized animals (Fig. 36A). Of note, despite absence of Core-specific T cell proliferation in the spleen, the number of IFNγ secreting T cells was significantly higher in the αDEC-205/Core immunized group when compared to control animals (αDEC-205) *(* $p = 0.0330$)* as revealed by ELISPOT analysis. Moreover, although not reaching the level of statistical significance, the number of splenic T cells from αDEC-205/Core immunized mice secreting IFNγ also clearly exceeded those detectable in mice vaccinated with the soluble Core antigen (Fig. 36B). Thus, these data indicate that DEC-205-mediated targeting of antigen to DCs more efficiently induces IFNγ producing Th1 effector cells than immunization with comparable amounts of soluble Core protein.

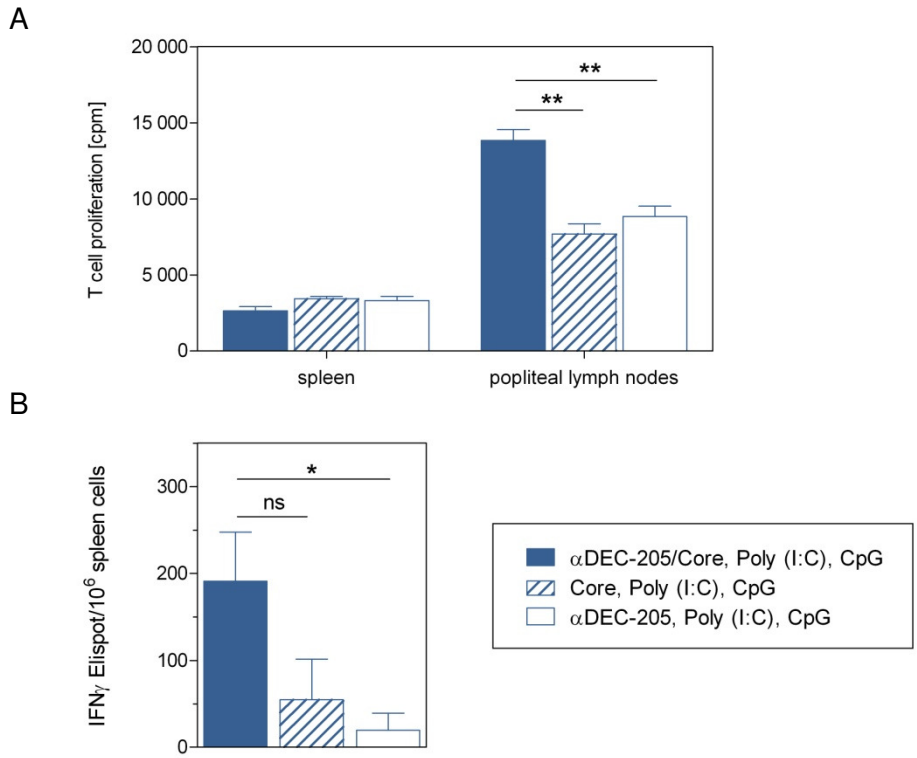

Figure 36. Cellular immune responses after αDEC-205/Core immunization.

Balb/c (n = 3) were immunized s.c. in the hind footpad with either 5 µg αDEC-205/Core, 5 µg αDEC-205 or 1.25 µg Core in addition to 50 µg Poly (I:C) and 50 µg CpG on day 0 and 7. One week later, the mice were sacrificed and ^{3}[H]-thymidine incorporation assay (A) as well as IFNγ ELISPOT analysis were performed. Data were expressed as means ± SEM (n = 3) and statistically analyzed by unpaired, two-tailed *t*-test. One independent experiment is displayed. A) MACS-isolated T cells from spleen and popliteal lymph nodes were co-cultured with BMDCs pulsed *in vitro* with 10 µg/ml of Core (aa 2-119). T cell proliferation was determined by [^{3}H] thymidine uptake in cpm *(** $P \leq 0.007$)*. B) Detection of IFNγ secreting splenocytes (1 x 10^{6} cells/ml) after re-stimulation with 5 µg/ml Core (aa 2-119) in triplicates. Results were expressed as spot forming units per 10^{6} cells with subtracted background derived from non-stimulated cells *(ns = 0.0813, * p = 0.0330)*. Abbreviations: bone-marrow derived cells (BMDCs); counts per minute (cpm); enzyme-linked immunosorbent spot (ELISPOT); magnetic activated cell sorting (MACS); standard error of the mean (SEM).

- ***Humoral immune responses***

In order to assess antibody response after this short-term immunization (14 days), concentration of total IgG in serum samples of vaccinated mice was evaluated by ELISA. Data obtained revealed relatively low Core-specific IgG levels in all groups tested (Fig 37A). Highest IgG levels were detectable in animals, which were immunized with soluble Core (OD_{450} < 0.5). In contrast, both, the conjugate and αDEC-205 immunized mice showed only background IgG levels suggesting that no Core-specific IgG was induced, which may be attributed to the short period between the first immunization and the analysis for IgG responses. When analyzing in more detail the Ig subtypes induced following αDEC-205/Core or soluble Core immunization of animals, levels of IgG_3 and IgM, which do both represent

early stage antibodies, were found to be equally high in both mouse groups (Fig. 37B). In addition and as seen before in NS3 immunization studies, not only IgG_1, but also IgG_{2a} antibody responses were induced in both, αDEC-205/Core and Core immunized mice to a similar extent.

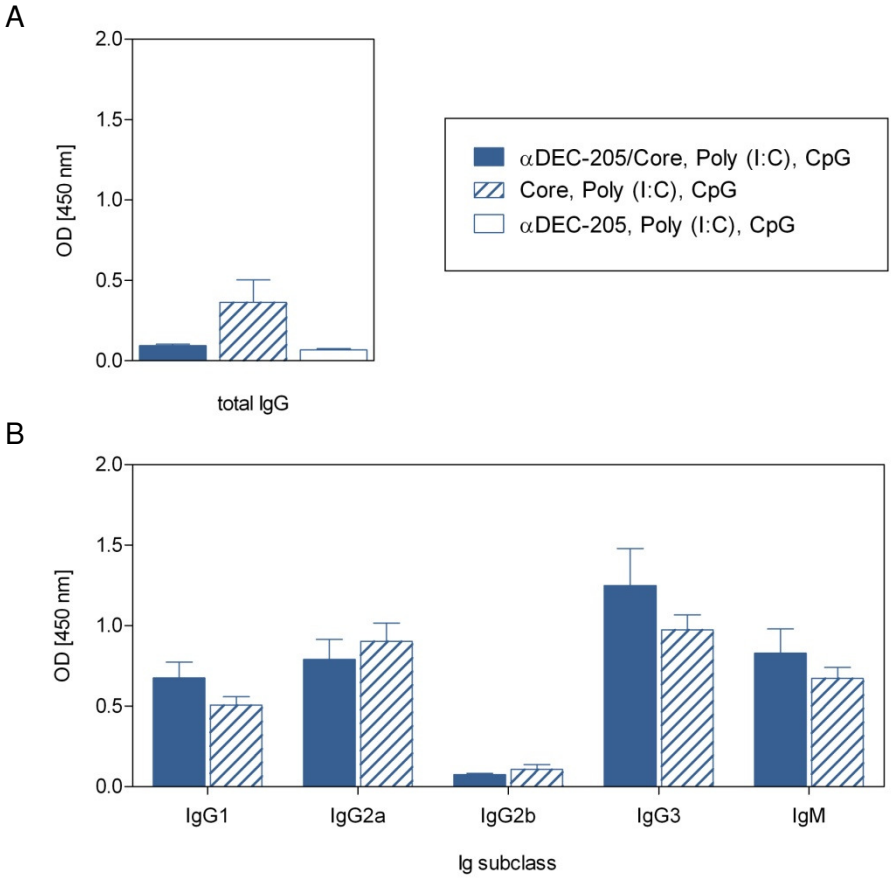

Figure 37. Humoral immune responses after αDEC-205/Core immunization.

Balb/c (n = 3) were immunized s.c. in the hind footpad with either 5 µg αDEC-205/Core, 5 µg αDEC-205 or 1.25 µg Core in addition to 50 µg Poly (I:C) and 50 µg CpG on day 0 and 7. One week later, the mice were sacrificed and total IgG (A) as well as Ig subclasses (B) were analyzed by ELISA. Data were expressed as means ± SEM (n = 3) and statistically compared by unpaired, two-tailed *t*-test. One independent experiment is displayed. A) Blood were collected at day 14 after first injection and sera diluted 1:500 were tested in ELISA plates, which had been coated with 2 ng/µl Core (aa 2-119) for detection of total IgG or B) IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3) and IgM. Bars represent means (n = 3) of the absorbance [OD_{450}] in duplicates. Abbreviations: enzyme-linked immunosorbent assay (ELISA); standard error of the mean (SEM).

Taken together, two times injection of αDEC-205/Core over 14 days led to significantly higher IFNγ release than in control or Core immunized mice. This was also consistent with increased levels of IgG_{2a} next to the early stage antibodies IgG_3 and IgM. Considering T cell proliferation only in popliteal lymph nodes draining the site of antigen application could be demonstrated, whereas the same failed in the periphery (spleen).

In summary, the purification of the HCV proteins NS3 (aa 1027-1218) (Fig. 25) and Core (aa 2-191) (Fig. 30) was successfully established. Despite considerable technical problems with the chemical conjugation of the self-made NS3 proteins to the DEC-205-specific antibody, successful generation of the conjugates consisting of αDEC-205 and purified Core (Fig. 31) or the recombinant NS3 (aa 1192-1459) purchased from a company (Fig. 26), respectively was achieved. Due to the fact that chemical crosslinking of both, Core and NS3 to the DEC-205 targeting antibody was associated with considerable loss of protein due to multiple experimental steps needed to generate conjugates pure enough for *in vivo* studies, immunization trials were severely limited by the low amount and concentration of αDEC-205/Core and αDEC-205/NS3. This was the reason why first immunization trials with the HCV protein conjugates were performed with far lower amounts of antigen than before in frame of the αDEC-205/OVA immunization trials known to induce robust antiviral immunity in the liver. Despite extremely low amounts of αDEC-205/NS3 and αDEC-205/Core conjugates used for vaccination, T cell proliferation induced following αDEC-205/NS3 immunization was found to be comparable to that observed in mice immunized with soluble NS3. However, one has to consider that the amount of soluble antigen was about 50-fold higher than that used in αDEC-205/NS3 immunized mice. Also, a significant increase in IFNγ producing T cells was found in the spleen of αDEC-205/Core but not Core immunized animals. Thus, provided that the technical problems regarding protein instability, chemical crosslinking and loss of protein and/or conjugate can be solved in the future, which would allow for the production of higher amounts of the conjugates, DEC-205-mediated antigen delivery to DCs may indeed represent a promising tool for prophylactic or therapeutic vaccination against HCV.

6 Discussion and future perspectives

Although vaccines have been successfully developed to protect against many infectious diseases, efficient vaccines are still lacking against persistent intracellular pathogens such as HIV and HCV. The design of vaccines has long time been reflected by the fact that “trial and error” has worked in many instances. In the past, vaccination approaches mostly focused on the induction of neutralizing antibodies. Current knowledge tends toward the development of optimized protocols that additionally include cellular components of the immune system. For the rational design of prophylactic or therapeutic vaccines against persistent viruses, cytotoxic $CD8^+$ T cells are of particular importance and there is an urgent need for the development of improved strategies to boost cellular immune responses in chronically infected patients. The pivotal role of DCs, which represent the most important cellular link between innate and adaptive immunity and the fact that DC-based immunotherapies can efficiently induce protective cellular and humoral immune responses, makes them a promising target for the future development of improved vaccination approaches (Berzofsky et al. 1999; Berzofsky et al. 2001; Banchereau & Palucka 2005; Tacken et al. 2007, Steinman & Banchereau 2007; Delamarre & Mellman 2011). Thus, the aim of this project was to compare two distinct *in vivo* DC targeting strategies with regard to their effectiveness in inducing both, humoral and cellular immunity, with special emphasis on the induction of antiviral immunity in the liver which is the target organ of HCV infection.

6.1 Analyzing the potential of DEC-205- vs. Toll-like receptor 2/6-mediated antigen delivery to dendritic cells with respect to HCV-specific immunotherapy

Previous investigations revealed that the TLR2/6 heterodimer agonist MALP-2 or its synthetic derivatives represent promising immunological tools applicable in the context of vaccination (Borsutzky et al. 2006), tumor therapy (Schneider et al. 2004), infection (Reppe et al. 2009) and airway inflammation (Weigt et al. 2005; Knothe et al. 2011). In particular, the recently developed synthetic derivative of MALP-2, either co-administered together with the antigen (OVA, BPPcysMPEG) or directly linked to the antigen (BPPcysOVAMPEG) seems to be most promising. Both derivatives target the antigen to the $CD8^+$ DC subset, which exhibits cross-priming activity resulting in the efficient induction of cytotoxic $CD8^+$ T cell-mediated immunity (Prajeeth et al. 2010). In addition, BPPcysMPEG has been demonstrated to exert various immunomodulatory activities in animal models (Cazorla et al. 2008; Schulze et al. 2008; Fuchs et al. 2010). Next to MALP-2 and its derivatives, the endocytosis receptor DEC-205 is considered as an exceptionally attractive target for immunotherapy, since it is

expressed at high levels on the surface of specifically those DCs (Inaba et al. 1995) that are capable of cross-presenting exogenous antigen via MHC-I molecules (Vremec & Shortman 1997; den Haan et al. 2000; Pooley et al. 2001; Iyoda et al. 2002). Moreover, DEC-205 targeting has also been successfully used for vaccination against viruses (Trumpfheller et al. 2006; Gurer et al. 2008), tumor antigens (Mahnke et al. 2005; Johnson et al. 2008) and bacterial pathogens (Do et al. 2010). In this context, several different approaches were developed to target antigens to DEC-205, either using the DEC-205 antibody chemically conjugated to the respective antigen (Bonifaz et al. 2002), in form of a recombinant antibody/antigen fusion protein (Hawiger et al. 2001) or as scFv (Johnson et al. 2008) (Tab. 3). For this thesis, the method of chemical conjugation was chosen to generate αDEC-205/antigen conjugates. Although this strategy was found to exhibit some technical limitations, especially when applied to antigens such as HCV NS3 that turned out to be instable in buffers that are compatible with optimal conditions for the chemical crosslinking reaction (5.2.1.2; 5.2.2.2), it comprises several advantages when compared to the other possible strategies which will be discussed below in more detail (6.2.3).

Encouraged by the previous observations that both, *in vivo* targeting of DCs via αDEC-205/antigen conjugates and the synthetic MALP-2 derivatives (BPPcysMPEG, BPPcysOVAMPEG), generally represent promising strategies to induce robust cellular immunity against a given antigen, the first aim of this thesis was to compare the two different DC targeting strategies, particularly with regard to the strength and nature of the cellular and humoral immune responses induced following immunization (Part I, 5.1). Since no published data exist so far on the outcome of *in vivo* targeting of antigen to DCs on the activation of protective T cell-mediated immunity in the liver, which is the place for HCV replication, special focus was laid on this particular organ. On the basis of the results obtained from the model antigen OVA, immunization trials were extended to the HCV proteins NS3 and Core in order to determine the suitability of the DEC-205-mediated targeting approach with respect to the future development of an immunotherapy against HCV infection (Part II, 5.2).

6.1.1 αDEC-205/OVA immunization results in efficient priming of antigen-specific CD4$^+$ and CD8$^+$ T cell responses

One critical factor decisive for the control of HCV infection in human patients is the induction of a strong, multi-specific and sustained HCV-specific CD4$^+$ and CD8$^+$ T cell response (Missale et al. 1996; Chang et al. 2001; Lechner et al. 2000; Grakoui et al. 2003; Bowen & Walker 2005). Since IFNγ is known to directly inhibit viral replication, an effective HCV vaccine should result in the induction of both, IFNγ secreting CD4$^+$ T helper cells (Th1) and CD8$^+$ CTLs (Frese et al. 2002). To rule out, whether the αDEC-205/OVA conjugate would be

superior to OVA, BPPcysMPEG or BPPcysOVAMPEG immunization with respect to the induction of IFNγ secreting effector T cells, ELISPOT (5.1.2.3) and *in vivo* CTL (5.1.2.2) assays were performed.

As already mentioned, $CD4^+$ T helper cells play a key role in cellular immunity, since they produce large amounts of IFNγ, exert cytolytic activity on MHC-II^+ target cells and sustain functional $CD8^+$ T cell memory (Bevan 2004; Casazza et al. 2006; Williams et al. 2006; Darrah et al. 2007). In addition, the activation of Th1 immunity may play a crucial role in the successful treatment of HCV infection (Tsai et al. 2003). The nature of the immune response following αDEC-205/OVA immunization appeared to be more Th1 than Th2 dominated as indicated by the secretion of IFNγ following antigen-specific *in vitro* stimulation of splenocytes (Fig. 19A). These results are consistent with previously published data demonstrating that *in vivo* targeting of antigen to DEC-205^+ DCs induces $CD4^+$ T helper cells to produce IFNγ indicative for a Th1 phenotype (Soares et al. 2007; Do et al. 2010). On the other hand, Th2 effector cells were also induced by targeting OVA to DEC-205 as indicated by IL-4 production following OVA-stimulation of splenocytes from αDEC-205/OVA immunized mice (Fig. 19B). Tsai et al. showed that spontaneous virus clearance is more likely to occur in acute hepatitis C patients that develop a Th1 profile (IFNγ and IL-2) than in patients developing a Th2 phenotype (IL-4 and IL-10) (Tsai et al. 1997). In addition, a correlation between Th1 responses and a less devastating inflammatory course of the disease in the chronic phase of the infection has been demonstrated by Woitas et al. supporting the beneficial role of Th1 cells for disease outcome (Woitas et al. 1997). Therefore, the Th1 phenotype induced by DEC-205-mediated antigen delivery to DCs is an important point with regard to the rational design of a HCV vaccine.

Since many lines of evidences demonstrate an important role of CTLs in the elimination of HCV during spontaneous viral clearance and in the context of HCV immunotherapy (Vertuani et al. 2002; Shoukry et al. 2003; Gremion & Cerny 2005), analysis of the induction of CTLs after immunization with αDEC-205/OVA was of particular importance. Besides $CD4^+$ effector T cells, also CTLs were efficiently primed already after the first αDEC-205/OVA immunization and strength of cytotoxicity further increased with the second and third αDEC-205/OVA injection (Fig. 18). Thus, DEC-205 targeting provides a feasible immunization strategy, since it efficiently induces $CD8^+$ T cell responses and moreover, $CD4^+$ T helper cells with a Th1 dominated phenotype (Bonifaz et al. 2002; Bonifaz et al. 2004; Bozzacco et al. 2010).

Regarding the TLR2/6 targeting strategy, no specific lysis was detectable after immunization with OVA, BPPcysMPEG (Fig. 18). This was unexpected, since it has been demonstrated before that injection of OVA together with BPPcysMPEG induces CTLs (Prajeeth et al. 2010). However, the antigen concentration used in that particular study by far exceeded the

one used in this thesis (3 mg OVA versus 7µg OVA). Therefore, the results obtained here, clearly indicate that targeting of OVA protein via DEC-205 is superior to the TLR2/6 targeting approach in priming CTLs, at least when low antigen concentrations are used for immunization. Furthermore, it clearly indicates that the MHC-I cross-presenting activity of DCs is particularly efficient following DEC-205 targeting. This might be due to two important conditions: First, the endocytosis receptor DEC-205 is known for its specialized function to deliver antigens to the late endosomes and to allow cross-presentation more efficiently than other DC targeting strategies. In this context, receptor-mediated antigen uptake was shown to result in up to 400 times more efficient MHC-I and MHC-II presentation of antigen than endocytosis of the soluble OVA protein (Mahnke et al. 2000; Bonifaz et al. 2002; Bonifaz et al. 2004; Bozzacco et al. 2010). Secondly, in mice DEC-205 is almost exclusively expressed on DCs (Inaba et al. 1995; Tacken et al. 2007), whereas BPPcysMPEG also binds to monocytes and macrophages, respectively (Rharbaoui et al. 2002; Link et al. 2004).

Analyzing the cytokine profile following immunization with OVA co-administered with BPPcysMPEG, it became apparent that primarily Th2 and less Th1 effector cells were induced. This was indicated by the significant IL-4 secretion after MHC-II OVA peptide re-stimulation, whereas the IFNγ release was significantly lower than in mice receiving αDEC-205/OVA (Fig. 19). Although Knothe et al. demonstrated a shift towards a Th1 cytokine profile in the lung following BPPcysMPEG-based immunization, data from Switalla and colleagues were in agreement with the results obtained here revealing no increase in IFNγ production by T cells when compared to immunization using LPS as DC maturation stimulus (Switalla et al. 2010; Knothe et al. 2011). In contrast, a mixed Th1/Th2 response has generally been observed in several vaccination trials using the adjuvant MALP-2 (Borsutzky et al. 2003; Rharbaoui et al. 2002; Rharbaoui et al. 2004), suggesting fundamental differences between the synthetic derivative BPPcysMPEG and MALP-2 in terms of supporting Th1 or Th2 differentiation.

Taken together, with regard to HCV immunotherapy, DEC-205-mediated targeting of OVA protein to DCs was found to be clearly superior to the BPPcysMPEG approach, since it resulted in the efficient priming of antigen-specific CTLs (Fig. 18) and IFNγ producing $CD4^+$ T cells (Fig. 19), both playing a vital role to overcome HCV liver infection.

Since Wille-Reece and colleagues have shown before that a TLR7/8 agonist directly conjugated to the HIV-1 Gag protein elicited stronger Th1 and $CD8^+$ T cell responses than separate co-administration of antigen and agonist, (Wille-Reece, Wu et al. 2005; Wille-Reece, Flynn et al. 2005), this vaccination strategy appeared to be promising also for inducing HCV-specific immunity. Indeed, targeting TLR2/6 via BPPcysOVAMPEG resulted in both, the induction of IFNγ and IL-4 secreting T cells (Fig. 19) and moreover, an effective

antigen-specific CTL response (Fig. 18) (Borsutzky et al. 2003; Rharbaoui et al. 2002; Rharbaoui et al. 2004). Thus, in contrast to BPPcysMPEG co-administered with OVA, both $CD4^+$ and $CD8^+$ T cell responses are effectively primed after immunization with BPPcysOVAMPEG, which clearly demonstrates its outstanding properties with respect to inducing broad and protective antiviral immunity, which was even stronger than in the αDEC-205/OVA immunized groups. However, it is important to emphasize that the BPPcysOVAMPEG construct comprises only the two immunodominant MHC-I and MHC-II OVA peptides instead of the entire OVA protein. Therefore, there would be a clear selection for T cells specifically responding against these particular epitopes. Moreover, when using comparable amount of BPPcysOVAMPEG and OVA, BPPcysMPEG for vaccination, the absolute number of immunodominant antigenic peptides available for MHC-I and MHC-II presentation is significantly higher in the BPPcysOVAMPEG construct than in the OVA, BPPcysMPEG preparation. One may speculate that in BPPcysOVAMPEG immunized animals the comparably high MHC-I and MHC-II peptide antigen concentration might be decisive for the induction of both, IFNγ and IL-4 producing $CD4^+$ T cells and the observed strong CTL response.

Despite the promising results obtained with the BPPcysOVAMPEG construct, administration of peptide antigens has obvious disadvantages in comparison to protein-based vaccines, in particular with regard to the development of a potent immunotherapy against HCV infection. Several data indicate that an early and robust immune response manifested by vigorous Th1, Th2, and multi-specific cellular immune responses to HCV proteins minimizes the genetic diversity of HCV quasispecies until the final variant is cleared (Gremion & Cerny 2005; Bowen & Walker 2005). Immunization with short peptides does not represent an optimal vaccination strategy as the use of peptide antigens is limited to small groups of individuals expressing the appropriate HLA allele capable of presenting the particular peptide. Moreover, since HCV is a quickly mutating virus and especially for this reason a lot of standard and nonstandard vaccination strategies failed in the past, an effective HCV vaccine should be based on protein antigens (Lechmann & Liang 2000; Leroux-Roels 2005; Manns et al. 2007). To induce a broad cellular immune response in the entire population, it is necessary that the candidate vaccine covers a large variety of potentially antigenic determinants presented by diverse MHC alleles. In addition, given the high degree of genetic heterogeneity of HCV, an effective vaccine should also be able to exert cross-protective immunity against various HCV genotypes, which is most likely not true for a peptide-based vaccine (Lechmann & Liang 2000). Thus, in light of the obvious disadvantages of peptide-based vaccines and the finding that OVA, BPPcycMPEG vaccination failed to induce CTLs and was found to be inferior to αDEC-205/OVA immunization in inducing IFNγ producing $CD4^+$ and $CD8^+$ effector T cell

responses, DEC-205-mediated targeting *in vivo* of antigen to DCs is considered the most useful approach for designing an HCV immunotherapy.

6.1.2 Cytotoxic T effector cells induced following αDEC-205/OVA immunization are capable to clear virus-infected hepatocytes

Since $CD8^+$ T cell activity accompanied by Th1 immunity is required to clear HCV infection (Liu et al. 2003; Tsai et al. 2003), it was important to study whether CTLs induced following immunization would be effective to clear virus-infected hepatocytes. Since the human HCV does not establish infection in mice, the recombinant adenovirus AdOVA-GFP-luc expressing in addition to GFP and luciferase the OVA as a surrogate antigen were used in order to study pathogen elimination from the liver following vaccination with αDEC-205/OVA, OVA, BPPcysMPEG and BPPcysOVAMPEG (5.1.3). These studies revealed that CTLs induced following αDEC-205/OVA and BPPcysOVAMPEG immunizations were able to recognize and kill virus-infected hepatocytes. This was indicated by the significant reduction of luciferase activity in the liver of the αDEC-205/OVA and BPPcysOVAMPEG immunized groups when compared to the luciferase activity in hepatocytes isolated from αDEC-205 and OVA, BPPcysMPEG immunized mice, in which no antigen-specific killing of hepatocytes was detectable (Fig. 24). These data were further confirmed by histological examination of the livers and results obtained from serum ALT analyses. Alterations in liver histology indicated hepatocellular necrosis and infiltrations of lymphocytes and eosinophils in αDEC-205/OVA and BPPcysOVAMPEG immunized mice, which provides an important indication for CTL-mediated hepatocyte lysis (Fig. 22). In addition, increased serum ALT levels as indicator for liver damage 6 days post infection confirmed antigen-specific killing of virus-infected cells (Fig. 23). Furthermore, FACS analysis revealed infiltration of $CD8^+$ T lymphocytes into the liver of αDEC-205/OVA and BPPcysOVAMPEG treated animals, suggesting that the observed liver damage was mediated by $IFN\gamma^+CD8^+$ T effector cells (Fig. 21). However, since it has not been formally proven that the liver infiltrating $CD8^+$ T cells produce IFNγ this needs to be determined in more detail in future studies.

Since a peptide-based vaccine such as BPPcysOVAMPEG has several shortcomings with regard to HCV immunotherapy for the reason mentioned above (6.1.1), special focus was laid in this thesis on the comparison of vaccination efficiency between αDEC-205/OVA and OVA, BPPcysMPEG. Although histological analysis of the liver revealed hepatocellular necrosis accompanied by lymphocyte infiltrations into the liver (Fig. 22) and moreover, a slight increase in serum ALT levels in OVA, BPPcysMPEG immunized mice (Fig. 23), this vaccination approach did not induce cellular immunity strong enough to eliminate virus-infected hepatocytes (Fig. 24). This was well in line with the failure to induce OVA-specific

CTLs as revealed by *in vivo* cytotoxic T cell assays (Fig. 18). Further studies are needed to univocal clarify the potential of BPPcycMPEG-mediated delivery of soluble protein antigens to DCs to induce antiviral immunity in the liver. Additional analyses intended to be performed in the future should combine the outstanding property of DEC-205-mediated antigen delivery to induce CTLs conferring antiviral activity in the liver with concomitant application of soluble OVA protein co-administered with either CpG and Poly (I:C) or BPPcysMPEG, which has not yet been done. Moreover, it should be analyzed whether immunization with soluble OVA protein, which is normally not accessible for the MHC-I processing pathway, would be cross-presented by DCs in the presence of Poly (I:C) and CpG (Datta et al. 2003; Schnurr et al. 2005; Schulz et al. 2005).

6.1.3 DEC-205-mediated targeting of antigen to dendritic cells induces vigorous and fast humoral immune responses

The role of humoral immunity in preventing and/or controlling HCV infection is not completely understood and remains controversial (Dustin & Rice 2006; Lang & Weiner 2008). Although neutralizing antibodies are generated during infection, several studies suggest that they have little effects on viral clearance and are not able to protect against HCV re-infection (Farci et al. 1992; Lai et al. 1994; Cooper et al. 1999; Pawlotsky 1999; Thimme et al. 2002). More recently, a positive correlation between the rapid induction of circulating neutralizing antibodies and viral clearance in acute phase patients has been shown, suggesting a contribution of the humoral immune response in the control of HCV infection (Lavillette et al. 2005; Pestka et al. 2007). Thus, a promising vaccination strategy against HCV should focus on the induction of a multi-specific and vigorous cellular host responses associated with multifaceted cross-neutralizing antibodies (Lechmann & Liang 2000; Houghton & Abrignani 2005; Inchauspé et al. 2007; Lang & Weiner 2008).

In order to evaluate the potency of the different *in vivo* DC targeting strategies to induce humoral immune responses to a given antigen, the kinetic of the IgG antibody responses was determined following vaccination. Interestingly, DEC-205-mediated targeting of antigen to DCs was found to induce the fastest and strongest IgG response when compared to BPPcysOVAMPEG or OVA, BPPcysMPEG immunization (Fig. 20). Thus, in addition to MHC-I cross-presentation of exogenous antigen accompanied with the induction of a strong CTL response (Fig. 18), DEC-205-mediated antigen delivery to DCs also efficiently facilitates the activation of $CD4^+$ T cells that provide B cell help. This is supported by the detection of IL-4 secreting Th2 cells in αDEC-205/OVA immunized mice (Fig. 19B) and data obtained here are well in line with the results published by Boscardin and colleagues, who showed that DEC-205-mediated antigen targeting was effective in eliciting T cell help for humoral immune responses (Boscardin et al. 2006). Apart from this, the serum IgG titers following

αDEC-205/OVA immunization were found to be stable over the period of the experiment and even slightly increased towards the end of the experiment. In contrast, targeting the whole protein to TLR2/6 via OVA, BPPcysMPEG immunization was far less efficient in inducing OVA-specific antibody responses than αDEC-205/OVA immunization, as indicated by a delayed and comparably low IgG titer found in this particular group of mice. Although immunization with BPPcysOVAMPEG turned out to induce the highest IgG titers after altogether three immunizations, the above mentioned disadvantages of peptide-based vaccines makes DEC-205-mediated antigen delivery to DCs superior to it with regard to the development of an effective vaccine against HCV.

Since an ideal vaccine should also to be able to stimulate memory B cell response to guarantee long term protection, challenge studies need to be performed in the future in order to further strengthen the potential of DEC-205 targeting as suitable tool for HCV immunotherapy.

6.1.4 The conflicting issue regarding the *in vivo* T cell proliferation following adoptive transfer into immunized mice

In order to assess the capacity of αDEC-205/OVA treatment as well as the other DC targeting strategies to induce adaptive immune responses CFSE-labeled OVA-specific $CD4^+$ and $CD8^+$ T cells were adoptively transferred into mice which were subsequently immunized with αDEC-205/OVA, OVA, BPPcysMPEG or BPPcysOVAMPEG. T cell proliferation was assessed in different compartments with special focus on the liver draining lymph nodes and the liver which represents the target organ for HCV infection.

In line with the results obtained from *in vivo* cytotoxicity assays (Fig. 18) and ELISPOT analyses (Fig. 19A), αDEC-205/OVA immunization was superior in inducing systemic $CD8^+$ T cell activation when compared with the other vaccination approaches (Fig. 16). However, whereas Bonifaz et al. showed vigorous proliferation of both antigen-specific $CD4^+$ and $CD8^+$ T cells in αDEC-205/OVA immunized mice after adoptive transfer, data obtained here revealed far less pronounced systemic expansion of $CD4^+$ T cells following αDEC-205/OVA treatment. One possible explanation for this discrepancy could be that the amount of antigen used for the immunization in this thesis was too low compared to the published data due to inefficient conjugation efficiency (Bonifaz et al. 2004; Johnson et al. 2008). Of note, in the liver-draining lymph nodes only DEC-205-mediated targeting of antigen to DCs induced expansion of $CD8^+$ T lymphocytes, further corroborating the unique potential of this approach to induce antigen-specific immunity in this particular compartment. However, no specific T cell proliferation was detectable in the liver itself, independent of the vaccination strategy used (Fig. 16). This is well in line with the current dogma that activated DCs usually migrate

to the lymph nodes, where they trigger adaptive immune responses and that T cell priming in the tolerogenic liver environment was described to result in T cell inactivation rather than activation (Cyster 1999; Bertolino et al. 2002, Thomson & Knolle 2010).

In contrast to αDEC-205/OVA treatment, OVA, BPPcysMPEG injection resulted in a very strong expansion of both antigen-specific $CD4^+$ and $CD8^+$ T cells. This effect was, however, restricted to the lymph nodes draining the site of OVA, BPPcysMPEG administration indicating a predominantly local and not a systemic effect of this vaccination approach (Fig. 16). Despite the observation that the percentage of $CD4^+$ and $CD8^+$ T cells in the liver-draining lymph nodes only marginally increased (Fig. 16), multiple rounds of cell division were detectable for both antigen-specific T cell subsets in the liver-draining lymph nodes of OVA, BPPcysMPEG immunized mice (Fig. 17). The same controversy was found for antigen-specific $CD4^+$ T cells in αDEC-205/OVA immunized mice (Fig. 17). Moreover, considering the immunization with BPPcysOVAMPEG only proliferation of OVA-specific $CD4^+$ T cells but not $CD8^+$ T cells was observed in the adoptive transfer experiments (Fig. 16, 17), despite the obvious existence of IFNγ producing effector CTLs in BPPcysOVAMPEG immunized mice that are capable of efficiently eliminating virus-infected hepatocytes (Fig. 18, 19, 24). Since the number of OVA-specific $CD4^+$ and $CD8^+$ T cells re-isolated for FACS analysis from adoptively transferred and immunized animals was comparably low, one may speculate that the discrepancy between results obtained from the different experiments may be related to this experimental limitation. Therefore, further investigations are needed to clarify this conflicting issue.

6.1.5 The role of the adjuvant for the specific outcome of the adaptive immune response following vaccination

Adjuvants are classically known to be essential to activate innate immunity and in particular to induce the maturation of antigen-presenting immune-stimulatory DCs (Pulendran & Ahmed 2006; Kool et al. 2008). For the development of DC-based immunotherapies, adjuvants are playing a key role, since they determine the type, magnitude and quality of the adaptive immune responses (Kanzler et al. 2007; Delamarre & Mellman 2011). Therefore, successful vaccine development requires in depth knowledge regarding how to achieve stable, safe and immunogenic vaccines and which adjuvants should be used to elicit the desired type of immune responses.

In this study, the αDEC-205/antigen conjugate was co-administered together with the TLR ligands Poly (I:C) and CpG, which are known to act via TLR3 and TLR9, respectively (Hemmi et al. 2000; Schulz et al. 2005). Ongoing clinical studies indicate that CpG is a relatively safe and well-tolerated adjuvant in humans, which is of course a critical point with respect to

clinical applications (Higgins et al. 2007; Bode et al. 2011). The same is true for Poly (I:C) making both adjuvants promising tools for vaccination of human patients (Trumpfheller et al. 2008; Butowski et al. 2009). Moreover, both TLR ligands induce Th1 immunity by predominantly inducing IFNγ-producing T cells, which is, as mentioned above, of special interest for the treatment of chronic viral infections such as HCV (Tsai et al. 1997; Woitas et al. 1997; Trumpfheller et al. 2008; Longhi et al. 2009; Stahl-Hennig et al. 2009; Bode et al. 2011). In addition, Poly (I:C) as well as CpG show a unique ability to promote cross-presentation of $CD8^+$ DCs. This is may be due to the activation of signaling pathways alternative or complementary to those involving MyD88 (Datta et al. 2003; Schulz et al. 2005). The Poly (I:C) and CpG promoted Th1-dominated immune responses and the improved cross-presenting activity of DCs are well in line with data obtained in this project (Fig. 18, 19; 6.1.1). As $CD8^+$ T cell-mediated cytotoxic activity accompanied by Th1 immunity are required for successful elimination of HCV (Liu et al. 2003; Tsai et al. 2003; Gremion & Cerny 2005), Poly (I:C) and CpG fulfill the basic requirements for an adjuvant suitable for the development of an efficient HCV vaccine. This is further strengthened by the facts that TLR3 agonists have been described before as attractive adjuvant for the immunization against viruses and moreover, TLR9 ligation was shown to be required for the control of viral infections (O´Neill et al. 2009; Duthie et al. 2011).

According to the literature BPPcysMPEG-based vaccination should result in Th1-dominated immune responses (Switalla et al. 2010; Knothe et al. 2011). This is in contrast to data obtained in frame of this thesis which revealed a Th2 phenotype following OVA, BPPcysMPEG immunization (6.1.1). While Knothe and colleagues co-administered the antigen together with 1 µg BPPcysMPEG (intranasally) and focused on the immune responses in the lung, 10 µg of the synthetic MALP-2 derivative was co-injected (s.c.) together with the OVA antigen here and the liver was the compartment of special immunological interest. One could speculate that the adjuvant dose, the route of administration as well as the immunological compartment investigated may account for the observed controversy. Nevertheless, the results obtained in this thesis pointed towards the induction of Th2-dominated immune responses using the synthetic MALP-2 derivative as an adjuvant. Thus, the adjuvant BPPcysMPEG appears to be inferior compared to Poly (I:C) and CpG with regard to the development of a potent immunotherapy against HCV

Several publications have been shown that certain TLRs can synergize with each other to enhance T cell mediated immune responses through synergistic activation of DCs and Zhu and colleagues demonstrated that this in fact holds true for the combination of Poly (I:C) and CpG (Gautier et al. 2005; Napolitani et al. 2005; Warger et al. 2006; Zheng et al. 2008; Zhu et al. 2008). Thus, the outstanding capacity of DEC-205-based vaccination to induce IFNγ

producing T effector cells capable of eliminating virus-infected liver cells may partially account for the synergistic action of Poly (I:C) and CpG. In addition, both together may be more efficient in inducing antiviral immunity when compared to the BPPcysMPEG approach, which is based on the use of a single TLR agonist. Moreover, investigators from the same group have recently shown that combining the TLR ligands Poly (I:C), CpG and MALP-2 greatly increased the protective capacity of an HIV vaccine in mice in comparison to a vaccine including only two of these TLR ligands. Thus, the strength of protection was not dependent on increased numbers of peptide-specific T cells, but rather on the improved quality of the T cell responses primarily by amplifying their functional avidity for the antigen, which was necessary for viral clearance (Zhu et al. 2010). Based on this observation, it appears tempting to combine DEC-205-mediated *in vivo* targeting of antigen to DCs with Poly (I:C), CpG and BPPcysMPEG in order determine whether the synergistic effect would also be obtained with respect to antiviral immunity in the liver.

6.2 DEC-205-mediated *in vivo* delivery of HCV antigens to dendritic cells: generation of the conjugates and first vaccination trials

As pointed out in the current literature DEC-205-mediated antigen-delivery to DCs triggers the full repertoire of adaptive immune responses required for an efficient vaccination against HCV infection, i.e. the induction of a strong, multi-specific and sustained HCV-specific T cell response associated with cross-neutralizing antibodies with wide-ranging specificities (Missale et al. 1996; Lechmann & Liang 2000; Chang et al. 2001; Houghton & Abrignani 2005; Inchauspé et al. 2007; Lang & Weiner 2008). Whereas no published data exist concerning the induction of pathogen-specific immunity in the liver, it could be demonstrated in frame of this thesis that DEC-205 targeting promotes effective priming of CTLs (Fig. 18) that are able to clear virus-infected hepatocytes (Fig. 24). Moreover, the significant IFNγ response following antigen-specific *in vitro* stimulation (Fig. 19) indicates the predominating Th1 phenotype following αDEC-205/antigen administration and in addition to the cell-mediated immune response a fast and vigorous antibody response was induced (Fig. 20). Thus, since the DEC-205 targeting strategy was identified as promising tool for vaccination against HCV infection, the findings obtained from the studies using the model antigen OVA provided an essential basis for the second part of the thesis, which was the establishment of a DEC-205-based immunotherapy against HCV (5.2).

6.2.1 The experimental obstacles to generate the αDEC-205/HCV antigen conjugates

In the current study, the HCV proteins NS3 (aa 1027-1218) and Core (aa 2-191) from HCV genotype 1b have been selected for the generation αDEC-205/HCV protein conjugates. These protein antigens were chosen because first of all both, NS3 and Core are highly conserved (Bukh et al. 1994; Yasui et al. 1998; Leroux-Roels 2005) and moreover, immunization with the Core protein was shown to induce Core-specific antibody as well as CTL responses and numerous CTL epitopes have been identified in the NS3 protein (Battegay et al. 1995; Cerny et al. 1995; Diepolder et al. 1997; Geissler et al. 1998; Inchauspé et al. 1997). Finally, they have been assessed before by other investigators as attractive candidates for HCV vaccines (Yu et al. 2004; Cao et al. 2011).

As described in the result section, protein purification and chemical cross-linking to the DEC-205 antibody turned out to be more complicated than initially expected. Extensive technical problems were faced concerning the protein stability, the amount/concentration of protein available following recombinant production and purification and the identification of a suitable buffer that would allow for chemical conjugation of the purified protein to the antibody (5.2.1.2; 5.2.2.2). With respect to protein purification, the protocols published by Vishnuvardhan et al. and Mihailova et al. built the basis for NS3 and Core purification, but needed to be extensively modified in order to match the optimal requirements for protein stability and the chemical conjugation to αDEC-205 (Vishnuvardhan et al. 1997; Mihailova et al. 2006). For example, Mihailova and colleagues suggested solving the Core protein in PBS, whereas data obtained in this study revealed instability of the HCV Core antigen in this particular buffer (Mihailova et al. 2006). Thus, in order to obtain high solubility of Core for the subsequent conjugation, the urea concentration and the pH of the elution buffer was step-wise adjusted to 1.5 M urea and pH 7 (5.2.2.1). The observed differences in the Core protein stability between data published by Mihailova et al. and those obtained in this thesis may be attributed to the different sizes of the Core fragments used (aa 1-98 versus aa 2-191). Since the Core protein contains a hydrophobic C-terminal part and a highly hydrophilic N-terminus, it is obvious that both the increased protein size and the altered characteristic of the molecule may account for the observed protein instability (McLauchlan et al. 2000). For the NS3 protein it turned out to be even more complicated to identify a buffer that would ensure high protein solubility on the one hand and provide optimal conditions for the conjugation to αDEC-205 on the other hand (5.2.1.1). Based on the protocol published by Vishnuvardhan and colleagues the NS3 protein should be stably soluble in a buffer containing 40 % glycerol and with acidic pH (pH 4) (Vishnuvardhan et al. 1997). However, since the high glycerol concentration and the low pH were incompatible with the requirements for the chemical

conjugation to the DEC-205 antibody 1.5 M urea was added and the pH was increased to the maximum of 6.5 to at least partially avoid precipitation of the NS3. Nevertheless, despite considerable evidence it was not possible to obtain high NS3 stability in a buffer suitable for the chemical cross-linking reaction and therefore, only very low amounts of NS3 protein could be yielded for the subsequent experiments. Thus, limitations in the availability of stable NS3 protein led to the inefficient chemical conjugation to αDEC-205 and in contrast to the Core protein, which could be successfully conjugated to αDEC-205 (Fig. 31) this problem could not be solved in frame of this thesis. It is valid to speculate that in addition to the difficulties with the buffer conditions the molecular structure of NS3 may account for the difficulties with chemical conjugation. This is supported by the fact that the NS3 purchased from a company (aa 1192-1459), which contains a different part of the HCV NS3 protein, could successfully by coupled to αDEC-205 (Fig. 26).

Since it was possible that either the chemical conjugation reaction *per se* or conformational changes in the DEC-205 antibody binding domain due to cross-linking with a relatively large protein fragment could negatively influence the binding capacity of the antibody, an important experimental step following chemical cross-linking was to verify that the αDEC-205/antigen conjugates remained their capability to bind to the endocytosis receptor DEC-205 on the surface of DCs. For this purpose, different protocols have been tested and modified on the basis of the current literature (Mahnke et al. 2005; Dissertation Storn 2008; Johnson et al. 2008). Johnson et al. described that pre-incubation of BMDCs with the mDEC-205/antigen-scFv led to impaired staining with secondary antibodies (Johnson et al. 2008). Since in this thesis the BMDCs were as well first incubated with the αDEC-205/Core and αDEC-205/NS3 conjugates to allow binding to DEC-205 on DCs followed by secondary staining for either αDEC-205 or the HCV proteins, conformational changes induced within the conjugate following DEC-205 binding may have made the potential secondary antibody binding sides inaccessible for efficient binding and detection. However, whether or not the αDEC-205/NS3 conjugate at least in part retained its capacity to bind to DEC-205 could not be conclusively determined in frame of this study. Of note, proper binding of the αDEC-205/Core conjugate could be definitely shown on the basis of FACS analysis, ELISA and Immunofluorescence microscopy (Fig. 33, 34, 35), underlining that in general the experimental approaches used here should be sensitive enough for detection of conjugate binding to DCs.

Despite the difficulties with proving unrestrained binding ability of the αDEC-205/HCV protein conjugates, both αDEC-205/NS3 and αDEC-205/Core, were used to immunize mice in order to study, if HCV-specific cellular and humoral immune responses could be induced (5.2.1.4; 5.2.2.5). Due to the technical limitation with protein purification and/or conjugation only 5 µg of the conjugates could be injected per mouse instead of 30 µg utilized for the

αDEC-205/OVA immunization. However, Bonifaz et al. showed that s.c. immunization with 5 µg OVA chemically conjugated to αDEC-205 induced detectable immune responses in different experimental settings (Bonifaz et al. 2004). Although Trumpfheller and colleagues also administered as few as 5 µg of αDEC-205/antigen, they used recombinant αDEC-205/antigen fusion proteins (Trumpfheller et al. 2006; Trumpfheller et al. 2008), which are considered to be more efficient than the chemical conjugates. This is due to the fact that the antigen is not randomly linked to any part of the antibody and moreover, the antibody to antigen ratio is always one which enables the use of highly reproducible antigen doses for vaccination. Thus, since it was not possible to guarantee optimal chemical conjugation efficiency resulting in a median binding of one NS3 or Core protein to one DEC-205 antibody molecule and moreover, since it couldn´t be ruled out that the conjugation would not interfere with the binding capacity of at least a part of the αDEC-205, it may be possible that the injection of as few as 5 µg αDEC-205/HCV protein conjugates was too low to induce overt immune responses. This assumption is well in line with a recent publication, in which the authors utilized 20 µg of a chemical αDEC-205/antigen conjugate to elicit antigen-specific immunity (Stylianou et al. 2011).

Targeting HCV proteins to DEC-205 results in the induction of antigen-specific immune responses. The efficiency of DEC-205-mediated targeting of antigens to DCs has been shown in a variety of different settings (Tab. 3). Upon others, its suitability as an attractive vaccination strategy against viral pathogens causing chronic disease such as HIV has also been demonstrated (Trumpfheller et al. 2006; Bozzacca et al. 2007; Trumpfheller et al. 2008; Bozzacca et al. 2010). However*, in vivo* targeting of HCV proteins to DCs utilizing the DEC-205 antibody has not yet been established and published. In order to study, whether the αDEC-205/NS3 and αDEC-205/Core conjugates would be effective in inducing cellular and humoral immune responses to the respective HCV antigens, mice were immunized with either αDEC-205/NS3 in addition to Poly (I:C) and αCD40 or αDEC-205/Core co-administered with Poly (I:C) and CpG (5.2.1.4; 5.2.2.5). Both adjuvant mixtures have been proven to be suitable for inducing adaptive immunity in the context of DEC-205-mediated antigen release to DCs (Boscardin et al. 2006; Trumpfheller et al. 2006; Johnson et al. 2008). Assessment of the cellular immune responses by *in vitro* ^{3}[H]-thymidine incorporation assay revealed that the extent of antigen-specific proliferating T cells isolated from the spleen and popliteal lymph nodes of αDEC-205/NS3 immunized mice was comparable to those of animals treated with the soluble NS3 protein (Fig. 28). Moreover, both the IgG_1 and IgG_{2a} antibody titers were increased following αDEC-205/NS3 as well as NS3 immunization, suggesting the induction of Th1 and Th2 effector cells upon vaccination (Fig. 29B). Of note, the absolute amount of applied antigen was 50-fold higher in case of NS3 protein injection

than in αDEC-205/NS3 immunized mice, since both groups of mice received a total amount of 5 µg protein, of which the far most protein amount consisted of the antibody in case of the conjugate. Therefore, taking into account these fundamental differences in the antigen dose used for vaccination, DEC-205 targeting has again demonstrated to be superior to immunization with soluble protein also in an HCV-related setting. Unfortunately, these promising results obtained during the first αDEC-205/NS3 vaccination experiment were not reproducible and several possible reasons may account for this. First of all, due to the limited amount of the αDEC-205/NS3 conjugate the time schedule established within the αDEC-205/OVA experiments had to be shortened, i.e. only two instead of three immunizations were performed. It is therefore conceivable that the second boost, which is missing in the αDEC-205/NS3 experiment, is of fundamental importance for successful priming of cellular immune responses being detectable by ^{3}[H]-thymidine incorporation assay. Secondly, for every independent vaccination experiment it was necessary to produce a new batch of the chemical conjugate due to the technical limitations in producing higher amounts of αDEC-205/NS3 (see above). Therefore, another element of uncertainty was the difficulty to achieve a consistent and efficient conjugation of the antigen to the DEC-205 antibody. In addition, it could be not excluded that the functionality of the conjugate generated for the subsequent experiments was impaired, since binding studies did not reveal clearly positive results. In conclusion, despite considerable efforts undertaken to optimize NS3 protein purification, increasing the protein stability and to increase the amount of functional αDEC-205/NS3 conjugate available for vaccination experiments, it was not possible to finally solve these experimental problems in order to clarify the potential of DEC-205-mediated targeting of NS3 to DCs to induce robust HCV-specific cellular immunity.

Concerning the αDEC-205/Core immunization, the experiment was performed according to a publication by Johnson et al. (Johnson et al. 2008). In contrast to NS3, the amount of antigen within the αDEC-205/Core conjugate was adjusted to soluble Core, i.e. 5 µg of the Core conjugate and 1.25 µg of soluble Core protein were administered to the mice. Importantly, Core-specific T cell proliferation was exclusively induced following αDEC-205/Core immunization but not in animals treated with soluble Core or αDEC-205 alone (Fig. 36A). However, antigen-specific T cell proliferation following *in vitro* re-stimulation was restricted to the popliteal lymph nodes draining the site of antigen application and was not observed in the spleen. The absence of systemic immune activation was unexpected since NS3-specific T cell proliferation was detectable in both the popliteal lymph nodes and the spleen following αDEC-205/NS3 immunization (Fig. 28). Moreover, although using a different experimental setup, Bonifaz et al. have demonstrated before that DEC-205 targeting results in systemic antigen presentation, which was also expected to be the case for the αDEC-205/Core

conjugate (Bonifaz et al. 2004). Despite the lack of detectable proliferation of Core-specific splenic T cells, the number of IFNγ producing effector T cells in the spleen was found to be clearly increased following αDEC-205/Core immunization (Fig. 36B), which may indicate that the ELISPOT assay is more sensitive than the ^{3}[H]-thymidine incorporation assay and particularly suitable for the detection of less pronounced immune responses. In addition, as already mentioned for the αDEC-205/NS3 conjugate, the amount of 5 µg αDEC-205/Core used for the immunization might be too low to induce cellular immune responses being easily traceable with standard immunological assays. However, it is valid to speculate that the administration of more than 5 µg of the αDEC-205/Core conjugate would lead to systemic antigen presentation and efficient induction of cellular immunity. This, however, needs to be formally proven in future experiments. Moreover, challenge studies using recombinant Core-expressing viruses to mimicked HCV liver infection in mice need to be performed in the future in order to assess the potential of DEC-205-mediated targeting of the Core protein to DCs to induce HCV-specific immunity in the liver of immunized mice.

6.2.2 Different strategies to target antigen to DEC-205 on dendritic cells: chemical conjugation vs. recombinant antibodies and single chain fragment variables

Facing so many experimental problems regarding the generation of functional αDEC-205/HCV protein conjugates by chemical conjugation (6.2.1) the question arose whether other *in vivo* DC targeting strategies would exist that might be easier to deal with. As already mentioned before, targeting protein antigen to the endocytosis receptor DEC-205 on DCs is superior to TLR2/6-mediated antigen delivery, since it was found to be superior in priming of both IFNγ secreting effector $CD4^{+}$ and $CD8^{+}$ T cells as well as humoral immune responses, which is absolutely needed for an efficient HCV immunotherapy (6.2). Apart from the chemical conjugation, αDEC-205/antigen constructs can be also generated as recombinant fusion proteins and scFv (Tab. 3) (Boscardin et al. 2006; Trumpfheller et al. 2006; Johnson et al. 2008; Birkholz et al. 2010). Compared to other targeting strategies such as the BPPcysMPEG-mediated targeting of TLR2/6, the advantage of the different tools for targeting DEC-205 are that the DEC-205 antibody, either in form of a chemical conjugate, a fusion protein or as a scFv, is highly specific for DCs which largely prevents unspecific uptake of the antigen by other cells than DCs. At the same time, the highly specific delivery of antigens to DCs implies that lower antigen dosages are needed in order to activate immune responses, which may be beneficial with regard to possible adverse reactions. Another advantage is, that the costs for *in vivo* targeting of antigens to DCs will be lower in comparison to other DC-based approaches such as the *in vitro* maturation of monocyte-derived DCs followed by *ex vivo* antigen loading and re-transfer to the patient,

which in contrast to the *in vivo* DEC-205 targeting needs to be custom-made for every individual (2.3.3) (Johnson et al. 2008; Tacken & Figdor et al. 2011). Improving cost-effectiveness is a crucial point for HCV immunotherapy based on the fact that currently existing treatment options are extremely cost-intensive (Manns et al. 2007). Since the chemical conjugation of HCV proteins to αDEC-205 turned out to have several shortcomings with respect to cross-linking efficiency and reproducibility, which would represent a major obstacle for clinical application, αDEC-205-scFv appear to be an attractive alternative, in particular with regard to clinical application. In contrast to the complete DEC-205 antibody used for chemical conjugation, the minimized αDEC-205-scFv facilitates not only the application of a well defined amount of antigen used for the treatment, but should also be easier to be produced at a large scale (Johnson et al. 2008). Nevertheless, chemical conjugation represents a broadly applicable method for the cross-linking of easy to handle protein antigens to the DEC-205 antibody (as demonstrated for αDEC-205/OVA), which for many antigens and many possible applications will be a very good alternative to scFv. Nevertheless, the cloning, production and testing of αDEC-205/NS3-scFv and αDEC-205/Core-scFv will be done in future experiments.

6.3 Hepatitis C virus and the challenges for vaccination

HCV is a remarkably successful pathogen, which establishes persistent infection in 80 % of those patients who contract it and has been proven to be especially difficult to treat as indicated by the fact that despite extensive investigations efficient HCV vaccines are still lacking. The success of the virus to escape immune surveillance of the infected host has been considered to be based on distinct factors, partially induced by the virus itself. Moreover, HCV is proposed to take advantages of the tolerogenic environment of the liver, the compartment in which the infection manifest (Dustin & Rice 2007; Lang & Weiner 2008; Stoll-Keller et al. 2009).

The liver is a vital organ that has a wide range of functions including metabolic functions in lipid, carbohydrate and protein generation as well as in the degradation of toxic and waste products. Based on its physiological functions the organ is permanently exposed to harmless antigens such as gut-derived nutrients and neo-antigens that arise by adduct formation of metabolic products during detoxification (Dustin & Rice 2007; Thomson & Knolle 2010). For this reasons, it is not surprising that T cell priming occurring within the liver results more likely in T cell inactivation, tolerance or apoptosis than in activation in order to maintain immunological tolerance and to protect the organ from unbeneficial immune responses (Bertolino et al. 2002; Crispe 2003). This issue seems to be extremely important in light of HCV infection and designing an adequate vaccine. On the one hand, the tolerogenic

environment provides cover for the virus to evade and prolong the delay of adaptive immune responses (Dustin & Rice 2007). On the other hand, the site of primary $CD8^+$ T cell activation may determine the outcome of immune responses (Racanelli & Manigold 2007). It has been shown that naïve $CD8^+$ T cells activated within the liver exhibited defective cytotoxic functions and shortened half-life, whereas naïve $CD8^+$ T cells activated within the lymph nodes were capable of mediating hepatitis indicating viral clearance (Bowen et al. 2004). This is further underlined by Halliday and colleagues who suggested that antiviral T cells primed in the periphery during vaccination could be of a "superior" quality to those primed in the liver (Halliday et al. 2011). Data presented in this thesis showed that following DEC-205-mediated targeting of antigen to DCs $CD8^+$ T cells proliferated vigorously in the liver-draining lymph nodes (Fig. 16). This suggests that the DEC-205-based vaccination strategy might be effective to induce HCV-specific CTLs in the liver-draining lymph nodes, which in turn would migrate to the liver to eliminate the virus. In addition, it is supported by the results obtained from the adenovirus challenge experiments which clearly demonstrated that virus-infected hepatocytes are effectively killed by CTLs despite the existence of a tolerogenic environment in the liver (Fig. 24).

Since HCV does not infect mouse hepatocytes, it could not be formally proven whether effective CTL priming via DEC-205 targeting would indeed result in clearance of HCV infected liver cells. During active viral replication there is a lack of viral antigen display on the surface of infected hepatocytes indicating viral antigen compartmentalization, which possibly accounts for a delayed T cell influx and activation in the liver (Pignatelli et al. 1986; Guidotti & Chisari 2006; Racanelli & Manigold 2007). More importantly, the mechanisms influenced by the HCV itself in order to blunt and evade antiviral immune responses play a crucial role. One mechanism is the rapid doubling time of the virus accompanied by the high mutation rate, which results in so-called quasispecies (2.4.2) (Kew et al. 2004). Consequently, the virus permanently tries to escape from the host immune responses with more or less success, so that in about 80 % of the patients acute HCV infection becomes persistent and turns to a chronic manifestation, whereas only in 10 - 30 % of the infected patients the virus is spontaneously eliminated (Fig. 11) (Woltman et al. 2010). Thus, it is conceivable that even though virus-specific CTLs are efficiently primed by a DEC-205-based vaccine, the virus may simultaneously evade host immunity. Apart from that, the HCV developed various strategies to manipulate and interfere with host adaptive immunity. For example, due to a complex interplay of immunological (e.g. T cell differentiation) and virological (e.g. ongoing antigen triggering) factors, exhaustion of HCV-specific $CD8^+$ T cells is induced during the course of chronic HCV infection (2.4.7) (Bengsch et al. 2010). Thus, although data obtained in this thesis revealed that DEC-205-mediated antigen release to DCs efficiently induces antiviral immunity in the liver, it cannot be excluded that viral escape mechanisms including T cell

exhaustion would finally counteract efficient elimination of the virus in αDEC-205/HCV antigen vaccinated individuals. However, it has been clearly demonstrated that an early and robust immune response manifested by vigorous and multi-specific T cell responses to HCV proteins not only minimizes the genetic diversity of HCV quasispecies, but also correlated with viral clearance during the acute phase of infection (Missale et al. 1996; Chang et al. 2001; Gremion & Cerny 2005; Bowen & Walker 2005). Thus, in principle protective antiviral immunity can be induced in infected patients, which is capable of controlling HCV infection. Results obtained in frame of this thesis underscored that a DEC-205-based vaccine induces the full repertoire of adaptive immune responses needed for an efficient vaccination against HCV infection, including the efficient induction of CTLs that are playing a pivotal role for virus elimination (Fig. 18, 24) accompanied by Th1 immunity (Fig. 19A) and a fast and vigorous antibody response (Fig. 20). Moreover, this vaccination strategy allows the usage of whole proteins, which is a crucial point with regard to inducing T and B cell responses against a broad repertoire of viral epitopes to minimize the risk of immune escape due to mutations in antigenic viral determinants (6.1.1) (Cooper et al., 1999; Lauer et al., 2004). Thus, *in vivo* targeting of HCV antigens to DCs via the DEC-205 endocytosis receptor may indeed represent a promising new approach to stimulate vigorous and multi-specific antiviral immune responses and to facilitate effective HCV clearance from the liver.

7 Appendix

7.1 Abbreviations

α	**alpha**
aa	amino acid
AAV	adeno-associated virus
ABTS	2,2`-azino-bis (3-ethylbenzothioazoline-6-sulfonic acid) diammonium salt
AEC	3-amino-9-ethyl-carbozole
ALT	alanine transaminase
APC	antigen-presenting cell
β	**beta**
BCR	B cell receptor
BDCA	blood dendritic cell antigen
BFA	brefeldin A
BMDC	bone-marrow-derived dendritic cell
BPPcysMPEG	S-[2,3-bispalmitoyiloxy-(2R)-propyl]-R-cysteinyl-amido-monomethoxyl polyethylene glycol
BPPcysOVAMPEG	BPPcysMPEG linked to OVA CD4 and CD8 peptide
BSA	bovine serum albumin
°C	**degree Celsius**
CCL-2	CC-chemokine ligand-2
CD	cluster of differentiation
CFSE	carboxyfluerescein diacetate succinimidyl ester
C_H	constant or C region of the heavy chain
C_L	constant or C region of the light chain
CLEC-1	C-type lectin receptor-1
CLP	common lymphoid progenitor
CLR	C-type lectin receptor
CMP	common myeloid progenitor
ConA	concanavalin A
CpG	cytosine-phosphate-guanine oligonucleotide sequences
cpm	counts per minute
CR	cysteine-rich domain
CRD	carbohydrate recognition domains

CSP	circumsporozoite protein
CTL	cytotoxic T lymphocyte
DC	**dendritic cell**
DCIR-2	dendritic cell inhibitory receptor 2
DC-SIGN	dendritic cell-specific ICAM-3 grabbing non-integrin
DLEC	dendritic cell lectin
DMF	N,N-Dimethylformamide
DMSO	dimethylsulfooxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
E. coli	***Escherichia coli***
EBNA1	Epstein-Barr virus nuclear antigen 1
EBV	Epstein-Barr virus
EDTA	ethylendieminetetraacetic acid
e.g.	*exempli gratia*
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
ER	endoplasmic reticulum
et al.	*et alii*
etc.	*et cetera*
FACS	**fluorescence-activated cell sorting**
FcR	Fc-receptor
FCS	fetal calf serum
Fig.	figure
FIRE	F4/80-like receptor
$Flt3^+$	FMS-related tyrosine kinase 3 ligand
FN	fibronectin type II repeat
γ	**gamma**
g	gram
GFP	green fluorescent protein
g/l	gram per liter
GST	glutathione S-transferase-tag

HAV	**hepatitis A virus**
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
H&E	hematoxylin and eosin
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRPO	horseradish peroxidase
HSC	hematopoietic stem cell
HSV	herpes simplex virus
HVR	hypervariable region
HZI	Helmholtz Centre for Infection Research
ICAM	**intercellular adhesion molecule**
IFN	interferon
Ig	immunoglobuline
IL	interleukin
IMDM	Iscove´s modified Dulbecco´s medium
IPTG	Isopropyl-,-D-thiogalactopyranosid
IRES	internal ribosome entry site
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
iTreg	induced Treg
i.v.	intravenously
kDa	**kilo Dalton**
l	**liter**
LDC	Langerhans dendritic cell
LPS	lipopolysaccharide
LY75	lymphocyte antigen 75
µ	**micro**
µl	microliter
m	milli
M	molar
mA	milliampere
mAb	monoclonal antibody
MACS	magnetic activated cell sorting

MALP-2	mycoplasma macrophage activating lipopeptide-2
mDC	myeloid dendritic cell
2-MEA	2-mercaptoethylamine HCl
mg	milligram
mg/ml	milligram per milliliter
MHC	major histocompatibility complex
MHC I	major histocompatibility complex class I molecules
MHC II	major histocompatibility complex class II molecules
MMR	macrophage mannose receptor
MR	mannose receptor
m/sec	meter per second
m/v	mass per volume
MWCO	molecular weight cut off
MyD88	myeloid differentiation primary response gene 88
NCR	**non-coding regions**
ng/µl	nanogram per microliter
NHS ester	N-hydroxysuccinimide ester
NK cells	natural killer cells
NKT cells	natural killer T cells
NLDC	non-lymphoid dendritic cells
nTreg	naturally occurring Treg
OD	**optical density**
OVA	ovalbumin
P	**proline-rich regions**
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PFA	paraformaldehyde
PFU	plaque forming units
PMA	phorbol 12-myristate 13-acetate
Poly (I:C)	polyinosine:polycytadilic acid
pre-DC	precursors of dendritic cells
PRR	pattern-recognition receptor
PVDF	polyvinylidene difluoride

RNA	**ribonucleic acid**
RT	room temperature
SARS	**severe acute respiratory syndrome**
s.c.	subcutaneously
scFv	single-chain Fragment variable
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
ss(+)RNA	positive strand ribonucleic acid virus
sulfo-SMCC	sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate
Tab.	**Table**
TBS	tris buffered saline
TCEP	tris(2-carboxyethyl)phosphine
TCR	T cell receptor
TGFβ	transforming growth factor β
Th	naïve $CD4^+$ T helper cells
thymic ECs	thymic endothelial cells
TLR	Toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNFα	tumor necrosis factor alpha
Treg	regulatory T cell
Tris-Base	Tris (hydroxymethyl)-aminomethane
Tris-HCl	Tris-(hydroxymethyl)-aminomethanhydrochlorid
TRP-2	tyrosinase-related protein
v/v	**volume per volume**
V_H	variable or V region of the heavy chain
V_L	variable or V region of the light chain
vs.	versus
WB	**western blot**
WHO	world health organization

7.2 Figures

7.3 Tables

7.4 References

Adler, A. J., Marsh, D. W., Yochum, G. S., Guzzo, J. L., Nigam, A., Nelson, W. G. and Pardoll, D. M. (1998). $CD4^+$ T cell tolerance to parenchymal self-antigens requires presentation by bone marrow-derived antigen-presenting cells. J. Exp. Med. 187: 1555-1564.

Ahmad, A. and Alvarez, F. (2004). Role of NK and NKT cells in the immunopathogenesis of HCV-induced hepatitis. J. Leukoc. Biol. 76: 743-759.

Akira, S. and Takeda, K. (2004). Toll-like receptor signalling. Nat. Rev. Immunol. 4: 499-511.

Alexopoulou, L., Holt, A. C., Medzhitov, R. and Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 413: 732-738.

Ardavin, C. (1997). Thymic dendritic cells. Immunol.Today. 18: 350-361.

Arnold-Schild, D., Hanau, D., Spehner, D., Schmid, C., Rammensee, H. G., de la, S. H. and Schild, H. (1999). Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. J. Immunol. 162: 3757-3760.

Bachem, A., Guttler, S., Hartung, E., Ebstein, F., Schaefer, M., Tannert, A., Salama, A., Movassaghi, K., Opitz, C., Mages, H. W., Henn, V., Kloetzel, P. M., Gurka, S. and Kroczek, R. A. (2010). Superior antigen cross-presentation and XCR1 expression define human $CD11c^+CD141^+$ cells as homologues of mouse CD8+ dendritic cells. J. Exp. Med. 207: 1273-1281.

Balan, V., Nelson, D. R., Sulkowski, M. S., Everson, G. T., Lambiase, L. R., Wiesner, R. H., Dickson, R. C., Post, A. B., Redfield, R. R., Davis, G. L., Neumann, A. U., Osborn, B. L., Freimuth, W. W. and Subramanian, G. M. (2006). A Phase I/II study evaluating escalating doses of recombinant human albumin-interferon-alpha fusion protein in chronic hepatitis C patients who have failed previous interferon-alpha-based therapy. Antivir. Ther. 11: 35-45.

Banchereau, J. and Palucka, A. K. (2005). Dendritic cells as therapeutic vaccines against cancer. Nat. Rev. Immunol. 5: 296-306.

Barber, D. L., Wherry, E. J., Masopust, D., Zhu, B., Allison, J. P., Sharpe, A. H., Freeman, G. J. and Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. Nature. 439: 682-687.

Bartenschlager, R., Frese, M. and Pietschmann, T. (2004). Novel insights into hepatitis C virus replication and persistence. Adv. Virus Res. 63: 71-180.

Bartosch, B., Bukh, J., Meunier, J. C., Granier, C., Engle, R. E., Blackwelder, W. C., Emerson, S. U., Cosset, F. L. and Purcell, R. H. (2003). In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. Proc. Natl. Acad. Sci. U.S.A. 100: 14199-14204.

Bartosch, B., Dubuisson, J. and Cosset, F. L. (2003). Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J. Exp. Med. 197: 633-642.

Battegay, M., Fikes, J., Di Bisceglie, A. M., Wentworth, P. A., Sette, A., Celis, E., Ching, W. M., Grakoui, A., Rice, C. M. and Kurokohchi, K. (1995). Patients with chronic hepatitis C have circulating cytotoxic T cells which recognize hepatitis C virus-encoded peptides binding to HLA-A2.1 molecules. J. Virol. 69: 2462-2470.

Bell, D., Young, J. W. and Banchereau, J. (1999). Dendritic cells. Adv. Immunol. 72: 255-324.

Bell, J. K., Botos, I., Hall, P. R., Askins, J., Shiloach, J., Segal, D. M. and Davies, D. R. (2005). The molecular structure of the Toll-like receptor 3 ligand-binding domain. Proc. Natl. Acad. Sci. U.S.A. 102: 10976-10980.

Bengsch, B., Seigel, B., Ruhl, M., Timm, J., Kuntz, M., Blum, H. E., Pircher, H. and Thimme, R. (2010). Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. PLoS. Pathog. 6: e1000947.

Bertolino, P., McCaughan, G. W. and Bowen, D. G. (2002). Role of primary intrahepatic T-cell activation in the 'liver tolerance effect'. Immunol. Cell Biol. 80: 84-92.

Berzofsky, J. A., Ahlers, J. D., Derby, M. A., Pendleton, C. D., Arichi, T. and Belyakov, I. M. (1999). Approaches to improve engineered vaccines for human immunodeficiency virus and other viruses that cause chronic infections. Immunol. Rev. 170: 151-172.

Berzofsky, J. A., Ahlers, J. D. and Belyakov, I. M. (2001). Strategies for designing and optimizing new generation vaccines. Nat. Rev. Immunol. 1: 209-219.

Berzofsky, J. A., Ahlers, J. D., Janik, J., Morris, J., Oh, S., Terabe, M. and Belyakov, I. M. (2004). Progress on new vaccine strategies against chronic viral infections. J. Clin. Invest. 114: 450-462.

Beutler, B. A. (2009). TLRs and innate immunity. Blood. 113: 1399-1407.

Bevan, M. J. (2004). Helping the CD8(+) T-cell response. Nat. Rev. Immunol. 4: 595-602.

Birkholz, K., Schwenkert, M., Kellner, C., Gross, S., Fey, G., Schuler-Thurner, B., Schuler, G., Schaft, N. and Dorrie, J. (2010). Targeting of DEC-205 on human dendritic cells results in efficient MHC class II-restricted antigen presentation. Blood. 116: 2277-2285.

Bode, C., Zhao, G., Steinhagen, F., Kinjo, T. and Klinman, D. M. (2011). CpG DNA as a vaccine adjuvant. Expert. Rev. Vaccines. 10: 499-511.

Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M. C. and Steinman, R. M. (2002). Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J. Exp. Med. 196: 1627-1638.

Bonifaz, L. C., Bonnyay, D. P., Charalambous, A., Darguste, D. I., Fujii, S., Soares, H., Brimnes, M. K., Moltedo, B., Moran, T. M. and Steinman, R. M. (2004). In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. J. Exp. Med. 199: 815-824.

Borsutzky, S., Fiorelli, V., Ebensen, T., Tripiciano, A., Rharbaoui, F., Scoglio, A., Link, C., Nappi, F., Morr, M., Butto, S., Cafaro, A., Muhlradt, P. F., Ensoli, B. and Guzman, C. A. (2003). Efficient mucosal delivery of the HIV-1 Tat protein using the synthetic lipopeptide MALP-2 as adjuvant. Eur. J. Immunol. 33: 1548-1556.

Borsutzky, S., Ebensen, T., Link, C., Becker, P. D., Fiorelli, V., Cafaro, A., Ensoli, B. and Guzman, C. A. (2006). Efficient systemic and mucosal responses against the HIV-1 Tat protein by prime/boost vaccination using the lipopeptide MALP-2 as adjuvant. Vaccine. 24: 2049-2056.

Boscardin, S. B., Hafalla, J. C., Masilamani, R. F., Kamphorst, A. O., Zebroski, H. A., Rai, U., Morrot, A., Zavala, F., Steinman, R. M., Nussenzweig, R. S. and Nussenzweig, M. C. (2006). Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses. J. Exp. Med. 203: 599-606.

Boulant, S., Montserret, R., Hope, R. G., Ratinier, M., Targett-Adams, P., Lavergne, J. P., Penin, F. and McLauchlan, J. (2006). Structural determinants that target the hepatitis C virus core protein to lipid droplets. J. Biol. Chem. 281: 22236-22247.

Bowen, D. G., Zen, M., Holz, L., Davis, T., McCaughan, G. W. and Bertolino, P. (2004). The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. J. Clin. Invest. 114: 701-712.

Bowen, D. G. and Walker, C. M. (2005). Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature. 436: 946-952.

Bozzacco, L., Trumpfheller, C., Siegal, F. P., Mehandru, S., Markowitz, M., Carrington, M., Nussenzweig, M. C., Piperno, A. G. and Steinman, R. M. (2007). DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to CD8+ T cells in a spectrum of human MHC I haplotypes. Proc. Natl. Acad. Sci. U.S.A. 104: 1289-1294.

Bozzacco, L., Trumpfheller, C., Huang, Y., Longhi, M. P., Shimeliovich, I., Schauer, J. D., Park, C. G. and Steinman, R. M. (2010). HIV gag protein is efficiently cross-presented when targeted with an antibody towards the DEC-205 receptor in Flt3 ligand-mobilized murine DC. Eur. J. Immunol. 40: 36-46.

Breckpot, K., Heirman, C., Neyns, B. and Thielemans, K. (2004). Exploiting dendritic cells for cancer immunotherapy: genetic modification of dendritic cells. J. Gene Med. 6: 1175-1188.

Brennan, B. (1998). Vaccines: the wave of the future. Perspect. Health. 3: 17-21.

Brocker, T., Riedinger, M. and Karjalainen, K. (1997). Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. J. Exp. Med. 185: 541-550.

Brown, R. S. (2005). Hepatitis C and liver transplantation. Nature. 436: 973-978.

Bruder, D., Westendorf, A. M., Hansen, W., Prettin, S., Gruber, A. D., Qian, Y., von, B. H., Mahnke, K. and Buer, J. (2005). On the edge of autoimmunity: T-cell stimulation by steady-state dendritic cells prevents autoimmune diabetes. Diabetes. 54: 3395-3401.

Bukh, J., Purcell, R. H. and Miller, R. H. (1994). Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. U.S.A. 91: 8239-8243.

Butler, M., Morel, A. S., Jordan, W. J., Eren, E., Hue, S., Shrimpton, R. E. and Ritter, M. A. (2007). Altered expression and endocytic function of CD205 in human dendritic cells, and detection of a CD205-DCL-1 fusion protein upon dendritic cell maturation. Immunology. 120: 362-371.

Butowski, N., Lamborn, K. R., Lee, B. L., Prados, M. D., Cloughesy, T., DeAngelis, L. M., Abrey, L., Fink, K., Lieberman, F., Mehta, M., Ian, R. H., Junck, L., Salazar, A. M. and Chang, S. M. (2009). A North American brain tumor consortium phase II study of poly-ICLC for adult patients with recurrent anaplastic gliomas. J. Neurooncol. 91: 183-189.

Caminschi, I., Lahoud, M. H. and Shortman, K. (2009). Enhancing immune responses by targeting antigen to DC. Eur. J. Immunol. 39: 931-938.

Cao, J., Chen, Z., Ren, Y., Luo, Y., Cao, M., Lu, W., Zhao, P. and Qi, Z. (2011). Oral immunization with attenuated Salmonella carrying a co-expression plasmid encoding the core and E2 proteins of hepatitis C virus capable of inducing cellular immune responses and neutralizing antibodies in mice. Vaccine. 29: 3714-3723.

Cãruntu, F. A. and Benea, L. (2006). Acute hepatitis C virus infection: Diagnosis, pathogenesis, treatment. J. Gastrointestin. Liver Dis. 15: 249-256.

Casazza, J. P., Betts, M. R., Price, D. A., Precopio, M. L., Ruff, L. E., Brenchley, J. M., Hill, B. J., Roederer, M., Douek, D. C. and Koup, R. A. (2006). Acquisition of direct antiviral effector functions by CMV-specific $CD4^+$ T lymphocytes with cellular maturation. J. Exp. Med. 203: 2865-2877.

Castellino, F., Boucher, P. E., Eichelberg, K., Mayhew, M., Rothman, J. E., Houghton, A. N. and Germain, R. N. (2000). Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J. Exp. Med. 191: 1957-1964.

Cavaleiro-Pinto, M., Peleteiro, B., Lunet, N. and Barros, H. (2011). Helicobacter pylori infection and gastric cardia cancer: systematic review and meta-analysis. Cancer Causes Control. 22: 375-387.

Cazorla, S. I., Frank, F. M., Becker, P. D., Corral, R. S., Guzman, C. A. and Malchiodi, E. L. (2008). Prime-boost immunization with cruzipain co-administered with MALP-2 triggers a protective immune response able to decrease parasite burden and tissue injury in an experimental Trypanosoma cruzi infection model. Vaccine. 26: 1999-2009.

Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A. and Colonna, M. (1999). Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat. Med. 5: 919-923.

Cerny, A., McHutchison, J. G., Pasquinelli, C., Brown, M. E., Brothers, M. A., Grabscheid, B., Fowler, P., Houghton, M. and Chisari, F. V. (1995). Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. J.Clin.Invest. 95: 521-530.

Chang, K. M., Thimme, R., Melpolder, J. J., Oldach, D., Pemberton, J., Moorhead-Loudis, J., McHutchison, J. G., Alter, H. J. and Chisari, F. V. (2001). Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. Hepatology. 33: 267-276.

Chaplin, D. D. (2010). Overview of the immune response. J. Allergy Clin. Immunol. 125: S3-23.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. and Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science. 244: 359-362.

Chow, V. T. (1993). Cancer and viruses. Ann. Acad. Med. Singapore. 22: 163-169.

Christie, J. M., Chapel, H., Chapman, R. W. and Rosenberg, W. M. (1999). Immune selection and genetic sequence variation in core and envelope regions of hepatitis C virus. Hepatology. 30: 1037-1044.

Colonna, M., Pulendran, B. and Iwasaki, A. (2006). Dendritic cells at the host-pathogen interface. Nat. Immunol. 7: 117-120.

Cools, N., Ponsaerts, P., Van, T., V and Berneman, Z. N. (2007). Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. J. Leukoc. Biol. 82: 1365-1374.

Cooper, S., Erickson, A. L., Adams, E. J., Kansopon, J., Weiner, A. J., Chien, D. Y., Houghton, M., Parham, P. and Walker, C. M. (1999). Analysis of a successful immune response against hepatitis C virus. Immunity. 10: 439-449.

Cresswell, P., Ackerman, A. L., Giodini, A., Peaper, D. R. and Wearsch, P. A. (2005). Mechanisms of MHC class I-restricted antigen processing and cross-presentation. Immunol. Rev. 207: 145-157.

Crispe, I. N. (2003). Hepatic T cells and liver tolerance. Nat. Rev. Immunol. 3: 51-62.

Curtsinger, J. M., Lins, D. C. and Mescher, M. F. (2003). Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. J. Exp. Med. 197: 1141-1151.

Cyster, J. G. (1999). Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs. J. Exp. Med. 189: 447-450.

Darrah, P. A., Patel, D. T., De Luca, P. M., Lindsay, R. W., Davey, D. F., Flynn, B. J., Hoff, S. T., Andersen, P., Reed, S. G., Morris, S. L., Roederer, M. and Seder, R. A. (2007). Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat. Med. 13: 843-850.

Datta, S. K., Redecke, V., Prilliman, K. R., Takabayashi, K., Corr, M., Tallant, T., DiDonato, J., Dziarski, R., Akira, S., Schoenberger, S. P. and Raz, E. (2003). A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. J. Immunol. 170: 4102-4110.

Davis, G. L., Balart, L. A., Schiff, E. R., Lindsay, K., Bodenheimer, H. C., Jr., Perrillo, R. P., Carey, W., Jacobson, I. M., Payne, J., Dienstag, J. L. and . (1989). Treatment of chronic hepatitis C with recombinant interferon alfa. A multicenter randomized, controlled trial. Hepatitis Interventional Therapy Group. N. Engl. J. Med. 321: 1501-1506.

De Luca, D. 'A. and Giraldi, G. (2011). A world wide public health problem: the principal re-emerging infectious diseases. Clin. Ter. 162: e93-e98.

Delamarre, L. and Mellman, I. (2011). Harnessing dendritic cells for immunotherapy.. Semin. Immunol. 23: 2-11.

den Haan, J. M., Lehar, S. M. and Bevan, M. J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J. Exp. Med. 192: 1685-1696.

Diepolder, H. M., Gerlach, J. T., Zachoval, R., Hoffmann, R. M., Jung, M. C., Wierenga, E. A., Scholz, S., Santantonio, T., Houghton, M., Southwood, S., Sette, A. and Pape, G. R. (1997). Immunodominant CD4+ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. J. Virol. 71: 6011-6019.

Dissertation Storn 2008:
http://archiv.ub.uni-heidelberg.de/volltextserver/volltexte/2008/8517/pdf/Dissertation_V.Storn.pdf

Do, Y., Koh, H., Park, C. G., Dudziak, D., Seo, P., Mehandru, S., Choi, J. H., Cheong, C., Park, S., Perlin, D. S., Powell, B. S. and Steinman, R. M. (2010). Targeting of LcrV virulence protein from Yersinia pestis to dendritic cells protects mice against pneumonic plague. Eur. J. Immunol. 40: 2791-2796.

Donahue, J. G., Munoz, A., Ness, P. M., Brown, D. E., Jr., Yawn, D. H., McAllister, H. A., Jr., Reitz, B. A. and Nelson, K. E. (1992). The declining risk of post-transfusion hepatitis C virus infection. N. Engl. J. Med. 327: 369-373.

Dranoff, G. (2004). Cytokines in cancer pathogenesis and cancer therapy. Nat. Rev. Cancer. 4: 11-22.

Dudziak, D., Kamphorst, A. O.., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H. W., Park, C. G., Steinman, R. M. and Nussenzweig, M. C. (2007). Differential antigen processing by dendritic cell subsets in vivo. Science. 315: 107-111.

Dustin, L. B. and Rice, C. M. (2007). Flying under the radar: the immunobiology of hepatitis C. Annu. Rev. Immunol. 25: 71-99.

Duthie, M. S., Windish, H. P., Fox, C. B. and Reed, S. G. (2011). Use of defined TLR ligands as adjuvants within human vaccines. Immunol. Rev. 239: 178-196.

Dzionek, A., Fuchs, A., Schmidt, P., Cremer, S., Zysk, M., Miltenyi, S., Buck, D. W. and Schmitz, J. (2000). BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J. Immunol. 165: 6037-6046.

Dzionek, A., Sohma, Y., Nagafune, J., Cella, M., Colonna, M., Facchetti, F., Gunther, G., Johnston, I., Lanzavecchia, A., Nagasaka, T., Okada, T., Vermi, W., Winkels, G., Yamamoto, T., Zysk, M., Yamaguchi, Y. and Schmitz, J. (2001). BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. J. Exp. Med. 194: 1823-1834.

East, L. and Isacke, C. M. (2002). The mannose receptor family. Biochim. Biophys. Acta. 1572: 364-386.

Ebensen, T., Link, C., Riese, P., Schulze, K., Morr, M. and Guzman, C. A. (2007). A pegylated derivative of alpha-galactosylceramide exhibits improved biological properties. J. Immunol. 179: 2065-2073.

Ebensen, T., Libanova, R., Schulze, K., Yevsa, T., Morr, M. and Guzman, C. A. (2011). Bis-(3',5')-cyclic dimeric adenosine monophosphate: Strong Th1/Th2/Th17 promoting mucosal adjuvant. Vaccine. 29: 5210-5220.

Edwards, A. D., Diebold, S. S., Slack, E. M., Tomizawa, H., Hemmi, H., Kaisho, T., Akira, S. and Reis e Sousa. (2003). Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. Eur. J. Immunol. 33: 827-833.

Erickson, A. L., Kimura, Y., Igarashi, S., Eichelberger, J., Houghton, M., Sidney, J., McKinney, D., Sette, A., Hughes, A. L. and Walker, C. M. (2001). The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. Immunity. 15: 883-895.

Evans, M. J., von, H. T., Tscherne, D. M., Syder, A. J., Panis, M., Wolk, B., Hatziioannou, T., McKeating, J. A., Bieniasz, P. D. and Rice, C. M. (2007). Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature. 446: 801-805.

Fanger, N. A., Wardwell, K., Shen, L., Tedder, T. F. and Guyre, P. M. (1996). Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells. J. Immunol. 157: 541-548.

Farci, P., Alter, H. J., Govindarajan, S., Wong, D. C., Engle, R., Lesniewski, R. R., Mushahwar, I. K., Desai, S. M., Miller, R. H., Ogata, N. and (1992). Lack of protective immunity against reinfection with hepatitis C virus. Science. 258: 135-140.

Figdor, C. G., van, K. Y. and Adema, G. J. (2002). C-type lectin receptors on dendritic cells and Langerhans cells. Nat. Rev. Immunol. 2: 77-84.

Figdor, C. G., de, V., I, Lesterhuis, W. J. and Melief, C. J. (2004). Dendritic cell immunotherapy: mapping the way. Nat. Med. 10: 475-480.

Flacher, V., Tripp, C. H., Stoitzner, P., Haid, B., Ebner, S., Del, F. B., Koch, F., Park, C. G., Steinman, R. M., Idoyaga, J. and Romani, N. (2010). Epidermal Langerhans cells rapidly capture and present antigens from C-type lectin-targeting antibodies deposited in the dermis. J. Invest Dermatol. 130: 755-762.

Foy, E., Li, K., Wang, C., Sumpter, R., Jr., Ikeda, M., Lemon, S. M. and Gale, M., Jr. (2003). Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science. 300: 1145-1148.

Frelin, L., Alheim, M., Chen, A., Soderholm, J., Rozell, B., Barnfield, C., Liljestrom, P. and Sallberg, M. (2003). Low dose and gene gun immunization with a hepatitis C virus nonstructural (NS) 3 DNA-based vaccine containing NS4A inhibit NS3/4A-expressing tumors in vivo. Gene Ther. 10: 686-699.

Frese, M., Schwarzle, V., Barth, K., Krieger, N., Lohmann, V., Mihm, S., Haller, O. and Bartenschlager, R. (2002). Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. Hepatology. 35: 694-703.

Frick, D. N. (2006). HCV Helicase: Structure, Function, and Inhibition.

Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marinos, G., Goncales, F. L., Jr., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J. and Yu, J. (2002). Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. 347: 975-982.

Fuchs, B., Knothe, S., Rochlitzer, S., Nassimi, M., Greweling, M., Lauenstein, H. D., Nassenstein, C., Muller, M., Ebensen, T., Dittrich, A. M., Krug, N., Guzman, C. A. and Braun, A. (2010). A Toll-like receptor 2/6 agonist reduces allergic airway inflammation in chronic respiratory sensitisation to Timothy grass pollen antigens. Int. Arch. Allergy Immunol. 152: 131-139.

Gasque, P. (2004). Complement: a unique innate immune sensor for danger signals. Mol. Immunol. 41: 1089-1098.

Gautier, G., Humbert, M., Deauvieau, F., Scuiller, M., Hiscott, J., Bates, E. E., Trinchieri, G., Caux, C. and Garrone, P. (2005). A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. J. Exp. Med. 201: 1435-1446.

Geijtenbeek, T. B., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Adema, G. J., van, K. Y. and Figdor, C. G. (2000). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell. 100: 575-585.

Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G. and van, K. Y. (2000). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell. 100: 587-597.

Geissler, M., Tokushige, K., Wakita, T., Zurawski, V. R., Jr. and Wands, J. R. (1998). Differential cellular and humoral immune responses to HCV core and HBV envelope proteins after genetic immunizations using chimeric constructs. Vaccine. 16: 857-867.

Grakoui, A., Shoukry, N. H., Woollard, D. J., Han, J. H., Hanson, H. L., Ghrayeb, J., Murthy, K. K., Rice, C. M. and Walker, C. M. (2003). HCV persistence and immune evasion in the absence of memory T cell help. Science. 302: 659-662.

Gremion, C. and Cerny, A. (2005). Hepatitis C virus and the immune system: a concise review. Rev. Med. Virol. 15: 235-268.

Grossmann, C., Tenbusch, M., Nchinda, G., Temchura, V., Nabi, G., Stone, G. W., Kornbluth, R. S. and Uberla, K. (2009). Enhancement of the priming efficacy of DNA vaccines encoding dendritic cell-targeted antigens by synergistic toll-like receptor ligands. BMC. Immunol. 10: 43.

Grouard, G., Rissoan, M. C., Filgueira, L., Durand, I., Banchereau, J. and Liu, Y. J. (1997). The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. J. Exp. Med. 185: 1101-1111.

Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C. and Amigorena, S. (2002). Antigen presentation and T cell stimulation by dendritic cells. Annu. Rev. Immunol. 20: 621-667.

Guidotti, L. G. and Chisari, F. V. (2006). Immunobiology and pathogenesis of viral hepatitis. Annu. Rev. Pathol. 1: 23-61.

Guo, M., Gong, S., Maric, S., Misulovin, Z., Pack, M., Mahnke, K., Nussenzweig, M. C. and Steinman, R. M. (2000). A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. Hum. Immunol. 61: 729-738.

Gurer, C., Strowig, T., Brilot, F., Pack, M., Trumpfheller, C., Arrey, F., Park, C. G., Steinman, R. M. and Munz, C. (2008). Targeting the nuclear antigen 1 of Epstein-Barr virus to the human endocytic receptor DEC-205 stimulates protective T-cell responses. Blood. 112: 1231-1239.

Haas, T., Metzger, J., Schmitz, F., Heit, A., Muller, T., Latz, E. and Wagner, H. (2008). The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. Immunity. 28: 315-323.

Halliday, J., Klenerman, P. and Barnes, E. (2011). Vaccination for hepatitis C virus: closing in on an evasive target. Expert. Rev. Vaccines. 10: 659-672.

Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M. and Weaver, C. T. (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat. Immunol. 6: 1123-1132.

Hart, D. N. (1997). Dendritic cells: unique leukocyte populations which control the primary immune response. Blood. 90: 3245-3287.

Hasan, U., Chaffois, C., Gaillard, C., Saulnier, V., Merck, E., Tancredi, S., Guiet, C., Briere, F., Vlach, J., Lebecque, S., Trinchieri, G. and Bates, E. E. (2005). Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J. Immunol. 174: 2942-2950.

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J. V., Steinman, R. M. and Nussenzweig, M. C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J. Exp. Med. 194: 769-779.

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M. and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 410: 1099-1103.

He, L. Z., Crocker, A., Lee, J., Mendoza-Ramirez, J., Wang, X. T.., Vitale, L. A., O'Neill, T., Petromilli, C., Zhang, H. F., Lopez, J., Rohrer, D., Keler, T. and Clynes, R. (2007). Antigenic targeting of the human mannose receptor induces tumor immunity. J. Immunol. 178: 6259-6267.

Heath, W. R., Belz, G. T., Behrens, G.. M., Smith, C. M., Forehan, S. P., Parish, I. A., Davey, G. M., Wilson, N. S., Carbone, F. R. and Villadangos, J. A. (2004). Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. Immunol. Rev. 199: 9-26.

Heller, T., Saito, S., Auerbach, J., Williams, T., Moreen, T. R., Jazwinski, A., Cruz, B., Jeurkar, N., Sapp, R., Luo, G. and Liang, T. J. (2005). An in vitro model of hepatitis C virion production. Proc. Natl. Acad. Sci. U.S.A. 102: 2579-2583.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. Nature. 408: 740-745.

Higgins, D., Marshall, J. D., Traquina, P., Van, N. G. and Livingston, B. D. (2007). Immunostimulatory DNA as a vaccine adjuvant. Expert. Rev. Vaccines. 6: 747-759.

Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M. and Shimotohno, K. (1991). Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc. Natl. Acad. Sci. U.S.A. 88: 5547-5551.

Hochrein, H., Shortman, K., Vremec, D., Scott, B., Hertzog, P. and O'Keeffe, M. (2001). Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. J. Immunol. 166: 5448-5455.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J. Immunol. 162: 3749-3752.

Houghton, M. and Abrignani, S. (2005). Prospects for a vaccine against the hepatitis C virus. Nature. 436: 961-966.

Idoyaga, J., Lubkin, A., Fiorese, C., Lahoud, M. H., Caminschi, I., Huang, Y., Rodriguez, A., Clausen, B. E., Park, C. G., Trumpfheller, C. and Steinman, R. M. (2011). Comparable T helper 1 (Th1) and CD8 T-cell immunity by targeting HIV gag p24 to CD8 dendritic cells within antibodies to Langerin, DEC205, and Clec9A. Proc. Natl. Acad. Sci. U.S.A. 108: 2384-2389.

Inaba, K., Swiggard, W. J., Inaba, M., Meltzer, J., Mirza, A., Sasagawa, T., Nussenzweig, M. C. and Steinman, R. M. (1995). Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. I. Expression on dendritic cells and other subsets of mouse leukocytes. Cell Immunol. 163: 148-156.

Inchauspé, G., Vitvitski, L., Major, M. E., Jung, G., Spengler, U., Maisonnas, M. and Trepo, C. (1997). Plasmid DNA expressing a secreted or a nonsecreted form of hepatitis C virus nucleocapsid: comparative studies of antibody and T-helper responses following genetic immunization. DNA Cell Biol. 16: 185-195.

Inchauspé, G. and Michel, M. L. (2007). Vaccines and immunotherapies against hepatitis B and hepatitis C viruses. J. Viral Hepat. 14 Suppl 1: 97-103.

Isaguliants, M. G., Petrakova, N. V., Mokhonov, V. V., Pokrovskaya, K., Suzdaltzeva, Y. G., Krivonos, A. V., Zaberezhny, A. D., Garaev, M. M., Smirnov, V. D. and Nordenfelt, E. (2003). DNA immunization efficiently targets conserved functional domains of protease and ATPase/helicase of nonstructural 3 protein (NS3) of human hepatitis C virus. Immunol. Lett. 88: 1-13.

Ito, T., Yang, M., Wang, Y. H., Lande, R., Gregorio, J., Perng, O. A., Qin, X. F., Liu, Y. J. and Gilliet, M. (2007). Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. J. Exp. Med. 204: 105-115.

Iyoda, T., Shimoyama, S., Liu, K., Omatsu, Y., Akiyama, Y., Maeda, Y., Takahara, K., Steinman, R. M. and Inaba, K. (2002). The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. J. Exp. Med. 195: 1289-1302.

Janeway, C. A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb. Symp. Quant. Biol. 54 Pt 1: 1-13.

Janeway, C. A., Jr., Travers P., Walport M. and Shlomchik M. J. (2001). Immunobiology, 5th edition. The Immune System in Health and Disease. Garland Science, New York.

Janeway, C. A., Jr., Travers P., Walport M. and Shlomchik M. J. (2005). Immunobiology, 6th edition. The Immune System in Health and Disease. Garland Science, New York.

Jelley-Gibbs, D. M., Strutt, T. M., McKinstry, K. K. and Swain, S. L. (2008). Influencing the fates of CD4 T cells on the path to memory: lessons from influenza. Immunol. Cell Biol. 86: 343-352.

Jiang, W., Swiggard, W. J., Heufler, C., Peng, M., Mirza, A., Steinman, R. M. and Nussenzweig, M. C. (1995). The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature. 375: 151-155.

Jin, M. S., Kim, S. E., Heo, J. Y., Lee, M. E., Kim, H. M., Paik, S. G., Lee, H. and Lee, J. O. (2007). Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell. 130: 1071-1082.

Johnson, T. S., Mahnke, K., Storn, V., Schonfeld, K., Ring, S., Nettelbeck, D. M., Haisma, H. J., Le, G. F., Kontermann, R. E. and Enk, A. H. (2008). Inhibition of melanoma growth by targeting of antigen to dendritic cells via an anti-DEC-205 single-chain fragment variable molecule. Clin. Cancer Res. 14: 8169-8177.

Jonuleit, H. and Schmitt, E. (2003). The regulatory T cell family: distinct subsets and their interrelations. J. Immunol. 171: 6323-6327.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F. and Liu, Y. J. (2001). Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. 194: 863-869.

Kalinski, P., Hilkens, C. M., Wierenga, E. A. and Kapsenberg, M. L. (1999). T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol. Today. 20: 561-567.

Kanzler, H., Barrat, F. J., Hessel, E. M. and Coffman, R. L. (2007). Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nat. Med. 13: 552-559.

Kaplan, D. H. (2010). In vivo function of Langerhans cells and dermal dendritic cells. Trends Immunol. 31: 446-451.

Kapsenberg, M. L. (2003). Dendritic-cell control of pathogen-driven T-cell polarization. Nat. Rev. Immunol. 3: 984-993.

Kariko, K., Ni, H., Capodici, J., Lamphier, M. and Weissman, D. (2004). mRNA is an endogenous ligand for Toll-like receptor 3. J. Biol. Chem. 279: 12542-12550.

Kato, M., Neil, T. K., Clark, G. J., Morris, C. M., Sorg, R. V. and Hart, D. N. (1998). cDNA cloning of human DEC-205, a putative antigen-uptake receptor on dendritic cells. Immunogenetics. 47: 442-450.

Kato, M., McDonald, K. J., Khan, S., Ross, I. L., Vuckovic, S., Chen, K., Munster, D., MacDonald, K. P. and Hart, D. N. (2006). Expression of human DEC-205 (CD205) multilectin receptor on leukocytes. Int. Immunol. 18: 857-869.

Kato, N., Ootsuyama, Y., Tanaka, T., Nakagawa, M., Nakazawa, T., Muraiso, K., Ohkoshi, S., Hijikata, M. and Shimotohno, K. (1992). Marked sequence diversity in the putative envelope proteins of hepatitis C viruses. Virus Res. 22: 107-123.

Keler, T., Ramakrishna, V. and Fanger, M. W. (2004). Mannose receptor-targeted vaccines. Expert. Opin. Biol. Ther. 4: 1953-1962.

Kew, M., Francois, G., Lavanchy, D., Margolis, H., Van, D. P., Grob, P., Hallauer, J., Shouval, D., Leroux-Roels, G. and Meheus, A. (2004). Prevention of hepatitis C virus infection. J. Viral Hepat. 11: 198-205.

Khoo, J. J., Forster, S. and Mansell, A. (2011). Toll-like receptors as interferon-regulated genes and their role in disease. J. Interferon Cytokine Res. 31: 13-25.

Klechevsky, E., Morita, R., Liu, M., Cao, Y., Coquery, S., Thompson-Snipes, L., Briere, F., Chaussabel, D., Zurawski, G., Palucka, A. K., Reiter, Y., Banchereau, J. and Ueno, H. (2008). Functional specializations of human epidermal Langerhans cells and $CD14^+$ dermal dendritic cells. Immunity. 29: 497-510.

Knothe, S., Mutschler, V., Rochlitzer, S., Winkler, C., Ebensen, T., Guzman, C. A., Hohlfeld, J., Braun, A. and Muller, M. (2011). Local treatment with BPPcysMPEG reduces allergic airway inflammation in sensitized mice. Immunobiology. 216: 110-117.

Kool, M., Soullie, T., van, N. M., Willart, M. A., Muskens, F., Jung, S., Hoogsteden, H. C., Hammad, H. and Lambrecht, B. N. (2008). Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J. Exp. Med. 205: 869-882.

Kraal, G., Breel, M., Janse, M. and Bruin, G. (1986). Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. J. Exp. Med. 163: 981-997.

Kronin, V., Wu, L., Gong, S., Nussenzweig, M. C. and Shortman, K. (2000). DEC-205 as a marker of dendritic cells with regulatory effects on CD8 T cell responses. Int. Immunol. 12: 731-735.

Kumar, H., Kawai, T. and Akira, S. (2009). Toll-like receptors and innate immunity. Biochem. Biophys. Res. Commun. 388: 621-625.

Kurokohchi, K., Akatsuka, T., Pendleton, C. D., Takamizawa, A., Nishioka, M., Battegay, M., Feinstone, S. M. and Berzofsky, J. A. (1996). Use of recombinant protein to identify a motif-negative human cytotoxic T-cell epitope presented by HLA-A2 in the hepatitis C virus NS3 region. J. Virol. 70: 232-240.

Kurts, C., Kosaka, H., Carbone, F.. R., Miller, J. F. and Heath, W. R. (1997). Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. J. Exp. Med. 186: 239-245.

Kurts, C., Heath, W. R., Carbone, F. R., Kosaka, H. and Miller, J. F. (1998). Cross-presentation of self antigens to CD8+ T cells: the balance between tolerance and autoimmunity. Novartis. Found. Symp. 215: 172-181.

Lai, M. E., Mazzoleni, A. P., Argiolu, F., De, V. S., Balestrieri, A., Purcell, R. H., Cao, A. and Farci, P. (1994). Hepatitis C virus in multiple episodes of acute hepatitis in polytransfused thalassaemic children. Lancet. 343: 388-390.

Lang, K. and Weiner, D. B. (2008). Immunotherapy for HCV infection: next steps. Expert. Rev. Vaccines. 7: 915-923.

Larosa, D. F. and Orange, J. S. (2008). 1. Lymphocytes. J. Allergy Clin. Immunol. 121: S364-S369.

Lauer, G. M. and Walker, B. D. (2001). Hepatitis C virus infection. N. Engl. J. Med. 345: 41-52.

Lauer, G. M., Barnes, E., Lucas, M., Timm, J., Ouchi, K., Kim, A. Y., Day, C. L., Robbins, G. K., Casson, D. R., Reiser, M., Dusheiko, G., Allen, T. M., Chung, R. T., Walker, B. D. and Klenerman, P. (2004). High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. Gastroenterology. 127: 924-936.

Lavillette, D., Morice, Y., Germanidis, G., Donot, P., Soulier, A., Pagkalos, E., Sakellariou, G., Intrator, L., Bartosch, B., Pawlotsky, J. M. and Cosset, F. L. (2005). Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. J. Virol. 79: 6023-6034.

Lechmann, M. and Liang, T. J. (2000). Vaccine development for hepatitis C. Semin. Liver Dis. 20: 211-226.

Lechner, F., Wong, D. K., Dunbar, P. R., Chapman, R., Chung, R. T., Dohrenwend, P., Robbins, G., Phillips, R., Klenerman, P. and Walker, B. D. (2000). Analysis of successful immune responses in persons infected with hepatitis C virus. J. Exp. Med. 191: 1499-1512.

Leonard, J. N., Ghirlando, R., Askins, J., Bell, J. K., Margulies, D. H., Davies, D. R. and Segal, D. M. (2008). The TLR3 signaling complex forms by cooperative receptor dimerization. Proc. Natl. Acad. Sci. U.S.A. 105: 258-263.

Leroux-Roels, G. (2005). Development of prophylactic and therapeutic vaccines against hepatitis C virus. Expert. Rev. Vaccines. 4: 351-371.

Libanova, R., Ebensen, T., Schulze, K., Bruhn, D., Norder, M., Yevsa, T., Morr, M. and Guzman, C. A. (2010). The member of the cyclic di-nucleotide family bis-(3', 5')-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant. Vaccine. 28: 2249-2258.

Lin, M. L., Zhan, Y., Villadangos, J. A. and Lew, A. M. (2008). The cell biology of cross-presentation and the role of dendritic cell subsets. Immunol. Cell Biol. 86: 353-362.

Link, C., Gavioli, R., Ebensen, T., Canella, A., Reinhard, E. and Guzman, C. A. (2004). The Toll-like receptor ligand MALP-2 stimulates dendritic cell maturation and modulates proteasome composition and activity. Eur. J. Immunol. 34: 899-907.

Liu, C., Zhu, H., Tu, Z., Xu, Y. L. and Nelson, D. R. (2003). CD8+ T-cell interaction with HCV replicon cells: evidence for both cytokine- and cell-mediated antiviral activity. Hepatology. 37: 1335-1342.

Liu, Y. J. (2005). IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annu. Rev. Immunol. 23: 275-306.

Longhi, M. P., Trumpfheller, C., Idoyaga, J., Caskey, M., Matos, I., Kluger, C., Salazar, A. M., Colonna, M. and Steinman, R.. M. (2009). Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J. Exp. Med. 206: 1589-1602.

Luzio, J. P., Parkinson, M. D., Gray, S. R. and Bright, N. A. (2009). The delivery of endocytosed cargo to lysosomes. Biochem. Soc. Trans. 37: 1019-1021.

Maamary, J., Array, F., Gao, Q., Garcia-Sastre, A., Steinman, R. M., Palese, P. and Nchinda, G. (2011). Newcastle disease virus expressing a dendritic cell-targeted HIV gag protein induces a potent gag-specific immune response in mice. J. Virol. 85: 2235-2246.

Macagno, A., Napolitani, G., Lanzavecchia, A. and Sallusto, F. (2007). Duration, combination and timing: the signal integration model of dendritic cell activation. Trends Immunol. 28: 227-233.

MacDonald, K. P., Munster, D. J., Clark, G. J., Dzionek, A., Schmitz, J. and Hart, D. N. (2002). Characterization of human blood dendritic cell subsets. Blood. 100: 4512-4520.

Maheshwari, A., Ray, S. and Thuluvath, P. J. (2008). Acute hepatitis C. Lancet. 372: 321-332.

Mahnke, K., Guo, M., Lee, S., Sepulveda, H., Swain, S. L., Nussenzweig, M. and Steinman, R. M. (2000). The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. J. Cell Biol. 151: 673-684.

Mahnke, K., Qian, Y., Knop, J. and Enk, A. H. (2003). Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells. Blood. 101: 4862-4869.

Mahnke, K., Qian, Y., Fondel, S., Brueck, J., Becker, C. and Enk, A. H. (2005). Targeting of antigens to activated dendritic cells in vivo cures metastatic melanoma in mice. Cancer Res. 65: 7007-7012.

Manns, M. P., McHutchison, J. G., Gordon, S. C., Rustgi, V. K.., Shiffman, M., Reindollar, R., Goodman, Z. D., Koury, K., Ling, M. and Albrecht, J. K. (2001). Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet. 358: 958-965.

Manns, M. P., Foster, G. R., Rockstroh, J. K., Zeuzem, S., Zoulim, F. and Houghton, M. (2007). The way forward in HCV treatment--finding the right path. Nat. Rev. Drug Discov. 6: 991-1000.

Manz, M. G., Traver, D., Miyamoto, T., Weissman, I. L. and Akashi, K. (2001). Dendritic cell potentials of early lymphoid and myeloid progenitors. Blood. 97: 3333-3341.

Martinon, F. and Tschopp, J. (2005). NLRs join TLRs as innate sensors of pathogens. Trends Immunol. 26: 447-454.

Matzinger, P. (1994). Tolerance, danger, and the extended family. Annu. Rev. Immunol. 12: 991-1045.

Matzinger, P. (2002). The danger model: a renewed sense of self. Science. 296: 301-305.

McGreal, E. P., Martinez-Pomares, L. and Gordon, S. (2004). Divergent roles for C-type lectins expressed by cells of the innate immune system. Mol. Immunol. 41: 1109-1121.

McGreal, E. P., Miller, J. L. and Gordon, S. (2005). Ligand recognition by antigen-presenting cell C-type lectin receptors. Curr. Opin. Immunol. 17: 18-24.

McHutchison, J. G., Gordon, S. C., Schiff, E. R., Shiffman, M. L., Lee, W. M., Rustgi, V. K., Goodman, Z. D., Ling, M. H., Cort, S. and Albrecht, J. K. (1998). Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N. Engl. J. Med. 339: 1485-1492.

McKay, P. F., Imami, N., Johns, M., Taylor-Fishwick, D. A., Sedibane, L. M., Totty, N. F., Hsuan, J. J., Palmer, D. B., George, A. J., Foxwell, B. M. and Ritter, M. A. (1998). The gp200-MR6 molecule which is functionally associated with the IL-4 receptor modulates B cell phenotype and is a novel member of the human macrophage mannose receptor family. Eur. J. Immunol. 28: 4071-4083.

McLauchlan, J. (2000). Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes. J. Viral Hepat. 7: 2-14.

Medzhitov, R. and Janeway, C. A., Jr. (1997). Innate immunity: the virtues of a nonclonal system of recognition. Cell. 91: 295-298.

Medzhitov, R. and Janeway, C., Jr. (2000). Innate immunity. N. Engl. J. Med. 343: 338-344.

Mellman, I. (1996). Endocytosis and molecular sorting. Annu. Rev. Cell Dev. Biol. 12: 575-625.

Mihailova, M., Fiedler, M., Boos, M., Petrovskis, I., Sominskaya, I., Roggendorf, M., Viazov, S. and Pumpens, P. (2006). Preparation of hepatitis C virus structural and non-structural protein fragments and studies of their immunogenicity. Protein Expr. Purif. 50: 43-48.

Missale, G., Bertoni, R., Lamonaca, V., Valli, A., Massari, M., Mori, C., Rumi, M. G., Houghton, M., Fiaccadori, F. and Ferrari, C. (1996). Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. J. Clin. Invest. 98: 706-714.

Mitchell, D. A. and Nair, S. K. (2000). RNA-transfected dendritic cells in cancer immunotherapy. J. Clin. Invest. 106: 1065-1069.

Miyanari, Y., Atsuzawa, K., Usuda, N., Watashi, K., Hishiki, T., Zayas, M., Bartenschlager, R., Wakita, T., Hijikata, M. and Shimotohno, K. (2007). The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. 9: 1089-1097.

Moradpour, D., Penin, F. and Rice, C. M. (2007). Replication of hepatitis C virus. Nat. Rev. Microbiol. 5: 453-463.

Mukherjee, S., Ghosh, R. N. and Maxfield, F. R. (1997). Endocytosis. Physiol Rev. 77: 759-803.

Mukhopadhyay, S., Herre, J., Brown, G. D. and Gordon, S. (2004). The potential for Toll-like receptors to collaborate with other innate immune receptors. Immunology. 112: 521-530.

Muzio, M., Bosisio, D., Polentarutti, N., D'amico, G., Stoppacciaro, A., Mancinelli, R., van't, V. C., Penton-Rol, G., Ruco, L. P., Allavena, P. and Mantovani, A. (2000). Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J. Immunol. 164: 5998-6004.

National Institutes of Health Consensus Development Conference Panel statement (1997). Management of hepatitis C. Hepatology. 26: 2S-10S.

Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F. and Lanzavecchia, A. (2005). Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat. Immunol. 6: 769-776.

Nchinda, G., Kuroiwa, J., Oks, M., Trumpfheller, C., Park, C. G., Huang, Y., Hannaman, D., Schlesinger, S. J., Mizenina, O., Nussenzweig, M. C., Uberla, K. and Steinman, R. M. (2008). The efficacy of DNA vaccination is enhanced in mice by targeting the encoded protein to dendritic cells. J. Clin. Invest. 118: 1427-1436.

Nchinda, G., Amadu, D., Trumpfheller, C., Mizenina, O., Uberla, K. and Steinman, R. M. (2010). Dendritic cell targeted HIV gag protein vaccine provides help to a DNA vaccine including mobilization of protective $CD8^+$ T cells. Proc. Natl. Acad. Sci. U.S.A. 107: 4281-4286.

Neumann-Haefelin, C., Blum, H. E., Chisari, F. V. and Thimme, R. (2005). T cell response in hepatitis C virus infection. J. Clin. Virol. 32: 75-85.

Neumann, A. U., Lam, N. P., Dahari, H., Gretch, D. R., Wiley, T. E., Layden, T. J. and Perelson, A. S. (1998). Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science. 282: 103-107.

Niebuhr, M., Muhlradt, P. F., Wittmann, M., Kapp, A. and Werfel, T. (2008). Intracutaneous injection of the macrophage-activating lipopeptide-2 (MALP-2) which accelerates wound healing in mice--a phase I trial in 12 patients. Exp. Dermatol. 17: 1052-1056.

O'Neill, L. A., Bryant, C. E. and Doyle, S. L. (2009). Therapeutic targeting of Toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacol. Rev. 61: 177-197.

Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L. and Aderem, A. (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc. Natl. Acad. Sci. U.S.A. 97: 13766-13771.

Pandey, K. N. (2009). Functional roles of short sequence motifs in the endocytosis of membrane receptors. Front Biosci. 14: 5339-5360.

Pawlotsky, J. M. (1999). Diagnostic tests for hepatitis C. J. Hepatol. 31 Suppl 1: 71-79.

Penin, F., Dubuisson, J., Rey, F. A., Moradpour, D. and Pawlotsky, J. M. (2004). Structural biology of hepatitis C virus. Hepatology. 39: 5-19.

Pereira, C. F., Torensma, R., Hebeda, K., Kretz-Rommel, A., Faas, S. J., Figdor, C. G. and Adema, G. J. (2007). In vivo targeting of DC-SIGN-positive antigen-presenting cells in a nonhuman primate model. J. Immunother. 30: 705-714.

Pestka, J. M., Zeisel, M. B., Blaser, E., Schurmann, P., Bartosch, B., Cosset, F. L., Patel, A. H., Meisel, H., Baumert, J., Viazov, S., Rispeter, K., Blum, H. E., Roggendorf, M. and Baumert, T. F. (2007). Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. Proc. Natl. Acad. Sci. U.S.A. 104: 6025-6030.

Petzold, C., Riewaldt, J., Koenig, T., Schallenberg, S. and Kretschmer, K. (2010). Dendritic cell-targeted pancreatic beta-cell antigen leads to conversion of self-reactive CD4(+) T cells into regulatory T cells and promotes immunotolerance in NOD mice. Rev. Diabet. Stud. 7: 47-61.

Piccioli, D., Tavarini, S., Borgogni, E., Steri, V., Nuti, S., Sammicheli, C., Bardelli, M., Montagna, D., Locatelli, F. and Wack, A. (2007). Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. Blood. 109: 5371-5379.

Pignatelli, M., Waters, J., Brown, D., Lever, A., Iwarson, S., Schaff, Z., Gerety, R. and Thomas, H. C. (1986). HLA class I antigens on the hepatocyte membrane during recovery from acute hepatitis B virus infection and during interferon therapy in chronic hepatitis B virus infection. Hepatology. 6: 349-353.

Pileri, P., Uematsu, Y., Campagnoli, S.., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D., Grandi, G. and Abrignani, S. (1998). Binding of hepatitis C virus to CD81. Science. 282: 938-941.

Plett, P. C. (2006). [Peter Plett and other discoverers of cowpox vaccination before Edward Jenner]. Sudhoffs Arch. 90: 219-232.

Plotkin S. L. and Plotkin S. A. in Plotkin S. A., Orenstein W. A. and Offit P. A. (2008). Vaccines 5th edition: A short history of vaccinations. Elsevier Health Sciences. ISBN 978-1-4160-3611-1.

Pooley, J. L., Heath, W. R. and Shortman, K. (2001). Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by $CD8^+$ dendritic cells. J. Immunol. 166: 5327-5330.

Post, J., Ratnarajah, S. and Lloyd, A. R. (2009). Immunological determinants of the outcomes from primary hepatitis C infection. Cell Mol. Life Sci. 66: 733-756.

Prajeeth, C. K., Jirmo, A. C., Krishnaswamy, J. K., Ebensen, T., Guzman, C. A., Weiss, S., Constabel, H., Schmidt, R. E. and Behrens, G. M. (2010). The synthetic TLR2 agonist BPPcysMPEG leads to efficient cross-priming against co-administered and linked antigens. Eur. J. Immunol. 40: 1272-1283.

Pulendran, B. and Ahmed, R. (2006). Translating innate immunity into immunological memory: implications for vaccine development. Cell. 124: 849-863.

Purcell, A. W., McCluskey, J. and Rossjohn, J. (2007). More than one reason to rethink the use of peptides in vaccine design. Nat.Rev.Drug Discov. 6: 404-414.

Racanelli, V. and Manigold, T. (2007). Presentation of HCV antigens to naive CD8+T cells: why the where, when, what and how are important for virus control and infection outcome. Clin. Immunol. 124: 5-12.

Radziewicz, H., Ibegbu, C. C., Fernandez, M. L., Workowski, K. A., Obideen, K., Wehbi, M., Hanson, H. L., Steinberg, J. P., Masopust, D., Wherry, E. J., Altman, J. D., Rouse, B. T., Freeman, G. J., Ahmed, R. and Grakoui, A. (2007). Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. J. Virol. 81: 2545-2553.

Ramakrishna, V., Treml, J. F., Vitale, L., Connolly, J. E., O'Neill, T., Smith, P. A., Jones, C. L., He, L. Z., Goldstein, J., Wallace, P. K., Keler, T. and Endres, M. J. (2004). Mannose receptor targeting of tumor antigen pmel17 to human dendritic cells directs anti-melanoma T cell responses via multiple HLA molecules. J. Immunol. 172: 2845-2852.

Randolph, G. J., Inaba, K., Robbiani, D. F., Steinman, R. M. and Muller, W. A. (1999). Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. Immunity. 11: 753-761.

Raney, K. D., Sharma, S. D., Moustafa, I. M. and Cameron, C. E. (2010). Hepatitis C virus non-structural protein 3 (HCV NS3): a multifunctional antiviral target. J. Biol. Chem. 285: 22725-22731.

Rehermann, B. and Nascimbeni, M. (2005). Immunology of hepatitis B virus and hepatitis C virus infection. Nat. Rev. Immunol. 5: 215-229.

Reizis, B., Bunin, A., Ghosh, H. S., Lewis, K. L. and Sisirak, V. (2011). Plasmacytoid dendritic cells: recent progress and open questions. Annu. Rev. Immunol. 29: 163-183.

Reppe, K., Tschernig, T., Luhrmann, A., van, L., V, Grote, K., Zemlin, M. V., Gutbier, B., Muller, H. C., Kursar, M., Schutte, H., Rosseau, S., Pabst, R., Suttorp, N. and Witzenrath, M. (2009). Immunostimulation with macrophage-activating lipopeptide-2 increased survival in murine pneumonia. Am. J. Respir. Cell Mol. Biol. 40: 474-481.

Rharbaoui, F., Drabner, B., Borsutzky, S., Winckler, U., Morr, M., Ensoli, B., Muhlradt, P. F. and Guzman, C. A. (2002). The Mycoplasma-derived lipopeptide MALP-2 is a potent mucosal adjuvant. Eur. J. Immunol. 32: 2857-2865.

Rharbaoui, F., Westendorf, A., Link, C., Felk, S., Buer, J., Gunzer, M. and Guzman, C. A. (2004). The Mycoplasma-derived macrophage-activating 2-kilodalton lipopeptide triggers global immune activation on nasal mucosa-associated lymphoid tissues. Infect. Immun. 72: 6978-6986.

Riedel, S. (2005). Edward Jenner and the history of smallpox and vaccination. Proc. (Bayl. Univ Med. Cent.). 18: 21-25.

Robbins, S. H., Walzer, T., Dembele, D., Thibault, C., Defays, A., Bessou, G., Xu, H., Vivier, E., Sellars, M., Pierre, P., Sharp, F. R., Chan, S., Kastner, P. and Dalod, M. (2008). Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. Genome Biol. 9: R17-

Robertson, B., Myers, G., Howard, C., Brettin, T., Bukh, J., Gaschen, B., Gojobori, T., Maertens, G., Mizokami, M., Nainan, O., Netesov, S., Nishioka, K., Shin i T, Simmonds, P., Smith, D., Stuyver, L. and Weiner, A. (1998). Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. Arch. Virol. 143: 2493-2503.

Sallusto, F., Cella, M., Danieli, C. and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J. Exp. Med. 182: 389-400.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R. M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R. and Vitelli, A. (2002). The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. 21: 5017-5025.

Schneider, C., Schmidt, T., Ziske, C., Tiemann, K., Lee, K. M., Uhlinsky, V., Behrens, P., Sauerbruch, T., Schmidt-Wolf, I. G., Muhlradt, P. F., Schmidt, J. and Marten, A. (2004). Tumour suppression induced by the macrophage activating lipopeptide MALP-2 in an ultrasound guided pancreatic carcinoma mouse model. Gut. 53: 355-361.

Schnurr, M., Chen, Q., Shin, A., Chen, W., Toy, T., Jenderek, C., Green, S.., Miloradovic, L., Drane, D., Davis, I. D., Villadangos, J., Shortman, K., Maraskovsky, E. and Cebon, J. (2005). Tumor antigen processing and presentation depend critically on dendritic cell type and the mode of antigen delivery. Blood. 105: 2465-2472.

Schulz, O., Diebold, S. S., Chen, M., Naslund, T. I., Nolte, M. A., Alexopoulou, L., Azuma, Y. T., Flavell, R. A., Liljestrom, P. and Reis e Sousa. (2005). Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature. 433: 887-892.

Schulze, K., Staib, C., Schatzl, H. M., Ebensen, T., Erfle, V. and Guzman, C. A. (2008). A prime-boost vaccination protocol optimizes immune responses against the nucleocapsid protein of the SARS coronavirus. Vaccine. 26: 6678-6684.

Seder, R. A. and Paul, W. E. (1994). Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu. Rev. Immunol. 12: 635-673.

Shakushiro, K., Yamasaki, Y., Nishikawa, M. and Takakura, Y. (2004). Efficient scavenger receptor-mediated uptake and cross-presentation of negatively charged soluble antigens by dendritic cells. Immunology. 112: 211-218.

Shi, Z., Cai, Z., Sanchez, A., Zhang, T., Wen, S., Wang, J., Yang, J., Fu, S. and Zhang, D. (2011). A novel Toll-like receptor that recognizes vesicular stomatitis virus. J. Biol. Chem. 286: 4517-4524.

Shortman, K. and Liu, Y. J. (2002). Mouse and human dendritic cell subtypes. Nat. Rev. Immunol. 2: 151-161.

Shortman, K. and Naik, S. H. (2007). Steady-state and inflammatory dendritic-cell development. Nat. Rev. Immunol. 7: 19-30.

Shoukry, N. H., Grakoui, A., Houghton, M., Chien, D. Y., Ghrayeb, J., Reimann, K. A. and Walker, C. M. (2003). Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. J. Exp. Med. 197: 1645-1655.

Shrimpton, R. E., Butler, M., Morel, A. S., Eren, E., Hue, S. S. and Ritter, M. A. (2009). CD205 (DEC-205): a recognition receptor for apoptotic and necrotic self. Mol. Immunol. 46: 1229-1239.

Siegal, F. P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P. A., Shah, K., Ho, S., Antonenko, S. and Liu, Y. J. (1999). The nature of the principal type 1 interferon-producing cells in human blood. Science. 284: 1835-1837.

Simmonds, P., Holmes, E. C., Cha, T. A., Chan, S. W., McOmish, F., Irvine, B., Beall, E., Yap, P. L., Kolberg, J. and Urdea, M. S. (1993). Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J. Gen. Virol. 74 (Pt 11): 2391-2399.

Simmonds, P., Bukh, J., Combet, C., Deleage, G., Enomoto, N., Feinstone, S., Halfon, P., Inchauspe, G., Kuiken, C., Maertens, G., Mizokami, M., Murphy, D. G., Okamoto, H., Pawlotsky, J. M., Penin, F., Sablon, E., Shin, I., Stuyver, L. J., Thiel, H. J., Viazov, S., Weiner, A. J. and Widell, A. (2005). Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology. 42: 962-973.

Sjogren, M. H., Sjogren, R., Holtzmuller, K., Winston, B., Butterfield, B., Drake, S., Watts, A., Howard, R. and Smith, M. (2005). Interferon alfacon-1 and ribavirin versus interferon alpha-2b and ribavirin in the treatment of chronic hepatitis C. Dig. Dis. Sci. 50: 727-732.

Snider, D. P., Kaubisch, A. and Segal, D. M. (1990). Enhanced antigen immunogenicity induced by bispecific antibodies. J. Exp. Med. 171: 1957-1963.

Soares, H., Waechter, H., Glaichenhaus, N., Mougneau, E., Yagita, H., Mizenina, O., Dudziak, D., Nussenzweig, M. C. and Steinman, R. M. (2007). A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo. J. Exp. Med. 204: 1095-1106.

Stahl-Hennig, C., Eisenblatter, M., Jasny, E., Rzehak, T., Tenner-Racz, K., Trumpfheller, C., Salazar, A. M., Uberla, K., Nieto, K., Kleinschmidt, J., Schulte, R., Gissmann, L., Muller, M., Sacher, A., Racz, P., Steinman, R. M., Uguccioni, M. and Ignatius, R. (2009). Synthetic double-stranded RNAs are adjuvants for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. PLoS. Pathog. 5: e1000373.

Steinman, L. (2007). A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. Nat. Med. 13: 139-145.

Steinman, R. M. and Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J. Exp. Med. 137: 1142-1162.

Steinman, R. M. and Swanson, J. (1995). The endocytic activity of dendritic cells. J. Exp. Med. 182: 283-288.

Steinman, R. M., Turley, S., Mellman, I. and Inaba, K. (2000). The induction of tolerance by dendritic cells that have captured apoptotic cells. J. Exp. Med. 191: 411-416.

Steinman, R. M. and Banchereau, J. (2007). Taking dendritic cells into medicine. Nature. 449: 419-426.

Stoll-Keller, F., Barth, H., Fafi-Kremer, S., Zeisel, M. B. and Baumert, T. F. (2009). Development of hepatitis C virus vaccines: challenges and progress. Expert. Rev. Vaccines. 8: 333-345.

Stylianou, E., Pepponi, I., van Dolleweerd, C. J., Paul, M. J., Ma, J. K. and Reljic, R. (2011). Exploring the vaccine potential of Dec-205 targeting in Mycobacterium tuberculosis infection in mice. Vaccine. 29: 2279-2286.

Switalla, S., Lauenstein, L., Prenzler, F., Knothe, S., Forster, C., Fieguth, H. G., Pfennig, O., Schaumann, F., Martin, C., Guzman, C. A., Ebensen, T., Muller, M., Hohlfeld, J. M., Krug, N., Braun, A. and Sewald, K. (2010). Natural innate cytokine response to immunomodulators and adjuvants in human precision-cut lung slices. Toxicol. Appl. Pharmacol.

Tacken, P. J., de, V., I, Torensma, R. and Figdor, C. G. (2007). Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. Nat. Rev. Immunol. 7: 790-802.

Tacken, P. J. and Figdor, C. G. (2011). Targeted antigen delivery and activation of dendritic cells in vivo: steps towards cost effective vaccines. Semin. Immunol. 23: 12-20.

Takahashi, H., Nakagawa, Y., Yokomuro, K. and Berzofsky, J. A. (1993). Induction of CD8+ cytotoxic T lymphocytes by immunization with syngeneic irradiated HIV-1 envelope derived peptide-pulsed dendritic cells. Int. Immunol. 5: 849-857.

Takahashi, M., Yamada, G., Miyamoto, R., Doi, T., Endo, H. and Tsuji, T. (1993). Natural course of chronic hepatitis C. Am. J. Gastroenterol. 88: 240-243.

Takeda, K. and Akira, S. (2004). Microbial recognition by Toll-like receptors. J. Dermatol. Sci. 34: 73-82.

Takeuchi, O., Kawai, T., Muhlradt, P. F., Morr, M., Radolf, J. D., Zychlinsky, A., Takeda, K. and Akira, S. (2001). Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int. Immunol. 13: 933-940.

Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R. L. and Akira, S. (2002). Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J. Immunol. 169: 10-14.

Tan, J. K. and O'Neill, H. C. (2005). Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. J. Leukoc. Biol. 78: 319-324.

Tarner, I. H. and Fathman, C. G. (2006). Does our current understanding of the molecular basis of immune tolerance predict new therapies for autoimmune disease? Nat. Clin. Pract. Rheumatol. 2: 491-499.

Taylor, K., Nguyen, A. and Stephenne, J. (2009). The need for new vaccines. Vaccine. 27 Suppl 6: G3-G8.

Tewari, K., Flynn, B. J., Boscardin, S. B., Kastenmueller, K., Salazar, A. M., Anderson, C. A., Soundarapandian, V., Ahumada, A., Keler, T., Hoffman, S. L., Nussenzweig, M. C., Steinman, R. M. and Seder, R. A. (2010). Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4+ T cell responses to Plasmodium falciparum circumsporozoite protein (CSP) and alphaDEC-CSP in non human primates. Vaccine. 28: 7256-7266.

Thimme, R., Oldach, D., Chang, K. M., Steiger, C., Ray, S. C. and Chisari, F. V. (2001). Determinants of viral clearance and persistence during acute hepatitis C virus infection. J. Exp. Med. 194: 1395-1406.

Thimme, R., Bukh, J., Spangenberg, H. C., Wieland, S., Pemberton, J., Steiger, C., Govindarajan, S., Purcell, R. H. and Chisari, F. V. (2002). Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. Proc. Natl. Acad. Sci. U.S.A. 99: 15661-15668.

Thomson, A. W. and Knolle, P. A. (2010). Antigen-presenting cell function in the tolerogenic liver environment. Nat. Rev. Immunol. 10: 753-766.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat. Rev. Immunol. 3: 133-146.

Trumpfheller, C., Finke, J. S., Lopez, C. B., Moran, T. M., Moltedo, B., Soares, H., Huang, Y., Schlesinger, S. J., Park, C. G., Nussenzweig, M. C., Granelli-Piperno, A. and Steinman, R. M. (2006). Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. J. Exp. Med. 203: 607-617.

Trumpfheller, C., Caskey, M., Nchinda, G., Longhi, M. P., Mizenina, O., Huang, Y., Schlesinger, S. J., Colonna, M. and Steinman, R. M. (2008). The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. Proc. Natl. Acad. Sci. U.S.A. 105: 2574-2579.

Tsai, S. L., Liaw, Y. F., Chen, M. H., Huang, C. Y. and Kuo, G. C. (1997). Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. Hepatology. 25: 449-458.

Tsai, S. L., Sheen, I. S., Chien, R. N., Chu, C. M., Huang, H. C., Chuang, Y. L., Lee, T. H., Liao, S. K., Lin, C. L., Kuo, G. C. and Liaw, Y. F. (2003). Activation of Th1 immunity is a common immune mechanism for the successful treatment of hepatitis B and C: tetramer assay and therapeutic implications. J. Biomed. Sci. 10: 120-135.

Tsuji, T., Matsuzaki, J., Kelly, M. P., Ramakrishna, V., Vitale, L., He, L. Z., Keler, T., Odunsi, K., Old, L. J., Ritter, G. and Gnjatic, S. (2011). Antibody-targeted NY-ESO-1 to mannose receptor or DEC-205 in vitro elicits dual human CD8+ and CD4+ T cell responses with broad antigen specificity. J. Immunol. 186: 1218-1227.

Turley, S. J., Inaba, K., Garrett, W. S., Ebersold, M., Unternaehrer, J., Steinman, R. M. and Mellman, I. (2000). Transport of peptide-MHC class II complexes in developing dendritic cells. Science. 288: 522-527.

Tuyaerts, S., Aerts, J. L., Corthals, J., Neyns, B., Heirman, C., Breckpot, K., Thielemans, K. and Bonehill, A. (2007). Current approaches in dendritic cell generation and future implications for cancer immunotherapy. Cancer Immunol. Immunother. 56: 1513-1537.

Underhill, D. M. and Ozinsky, A. (2002). Toll-like receptors: key mediators of microbe detection. Curr. Opin. Immunol. 14: 103-110.

Valladeau, J., Caux, C., Lebecque, S. and Saeland, S. (2001). [Langerin: a new lectin specific for Langerhans cells induces the formation of Birbeck granules]. Pathol. Biol. (Paris). 49: 454-455.

Valladeau, J., Clair-Moninot, V., zutter-Dambuyant, C., Pin, J. J., Kissenpfennig, A., Mattei, M. G., it-Yahia, S., Bates, E. E., Malissen, B., Koch, F., Fossiez, F., Romani, N., Lebecque, S. and Saeland, S. (2002). Identification of mouse langerin/CD207 in Langerhans cells and some dendritic cells of lymphoid tissues. J. Immunol. 168: 782-792.

Van Tendeloo, V, Snoeck, H. W., Lardon, F., Vanham, G. L., Nijs, G., Lenjou, M., Hendriks, L., Van, B. C., Moulijn, A., Rodrigus, I., Verdonk, P., Van Bockstaele, D. R. and Berneman, Z. N. (1998). Nonviral transfection of distinct types of human dendritic cells: high-efficiency gene transfer by electroporation into hematopoietic progenitor- but not monocyte-derived dendritic cells. Gene Ther. 5: 700-707.

Van Tendeloo, V, Ponsaerts, P., Lardon, F., Nijs, G., Lenjou, M., Van, B. C., Van Bockstaele, D. R. and Berneman, Z. N. (2001). Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood. 98: 49-56.

Vasselon, T. and Detmers, P. A. (2002). Toll receptors: a central element in innate immune responses. Infect. Immun. 70: 1033-1041.

Vercammen, E., Staal, J. and Beyaert, R. (2008). Sensing of viral infection and activation of innate immunity by toll-like receptor 3. Clin. Microbiol. Rev. 21: 13-25.

Verhasselt, V., Vosters, O., Beuneu, C., Nicaise, C., Stordeur, P. and Goldman, M. (2004). Induction of FOXP3-expressing regulatory CD4pos T cells by human mature autologous dendritic cells. Eur. J. Immunol. 34: 762-772.

Vertuani, S., Bazzaro, M., Gualandi, G., Micheletti, F., Marastoni, M., Fortini, C., Canella, A., Marino, M., Tomatis, R., Traniello, S. and Gavioli, R. (2002). Effect of interferon-alpha therapy on epitope-specific cytotoxic T lymphocyte responses in hepatitis C virus-infected individuals. Eur. J. Immunol. 32: 144-154.

Villadangos, J. A. and Heath, W. R. (2005). Life cycle, migration and antigen presenting functions of spleen and lymph node dendritic cells: limitations of the Langerhans cells paradigm. Semin. Immunol. 17: 262-272.

Villadangos, J. A. and Young, L. (2008). Antigen-presentation properties of plasmacytoid dendritic cells. Immunity. 29: 352-361.

Vishnuvardhan, D., Kakiuchi, N., Urvil, P. T., Shimotohno, K., Kumar, P. K. and Nishikawa, S. (1997). Expression of highly active recombinant NS3 protease domain of hepatitis C virus in E. coli. FEBS Lett. 402: 209-212.

Vremec, D. and Shortman, K. (1997). Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. J. Immunol. 159: 565-573.

Vremec, D., Pooley, J., Hochrein, H., Wu, L. and Shortman, K. (2000). CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. J. Immunol. 164: 2978-2986.

Warger, T., Osterloh, P., Rechtsteiner, G., Fassbender, M., Heib, V., Schmid, B., Schmitt, E., Schild, H. and Radsak, M. P. (2006). Synergistic activation of dendritic cells by combined Toll-like receptor ligation induces superior CTL responses in vivo. Blood. 108: 544-550.

Wasley, A. and Alter, M. J. (2000). Epidemiology of hepatitis C: geographic differences and temporal trends. Semin. Liver Dis. 20: 1-16.

Watts, C. (1997). Capture and processing of exogenous antigens for presentation on MHC molecules. Annu. Rev. Immunol. 15: 821-850.

Weigt, H., Nassenstein, C., Tschernig, T., Muhlradt, P. F., Krug, N. and Braun, A. (2005). Efficacy of macrophage-activating lipopeptide-2 combined with interferon-gamma in a murine asthma model. Am. J. Respir. Crit Care Med. 172: 566-572.

Wille-Reece, U., Wu, C. Y., Flynn, B. J., Kedl, R. M. and Seder, R. A. (2005). Immunization with HIV-1 Gag protein conjugated to a TLR7/8 agonist results in the generation of HIV-1 Gag-specific Th1 and CD8+ T cell responses. J. Immunol. 174: 7676-7683.

Wille-Reece, U., Flynn, B. J., Lore, K., Koup, R. A., Kedl, R. M., Mattapallil, J. J., Weiss, W. R., Roederer, M. and Seder, R. A. (2005). HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. Proc. Natl. Acad. Sci. U.S.A. 102: 15190-15194.

Williams, M. A., Tyznik, A. J. and Bevan, M. J. (2006). Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. Nature. 441: 890-893.

Wintergerst, E., Manz-Keinke, H., Plattner, H. and Schlepper-Schafer, J. (1989). The interaction of a lung surfactant protein (SP-A) with macrophages is mannose dependent. Eur. J. Cell Biol. 50: 291-298.

Witmer-Pack, M. D., Swiggard, W. J., Mirza, A., Inaba, K. and Steinman, R. M. (1995). Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. II. Expression in situ in lymphoid and nonlymphoid tissues. Cell Immunol. 163: 157-162.

Woitas, R. P., Lechmann, M., Jung, G., Kaiser, R., Sauerbruch, T. and Spengler, U. (1997). CD30 induction and cytokine profiles in hepatitis C virus core-specific peripheral blood T lymphocytes. J. Immunol. 159: 1012-1018.

Woltman, A. M., Boonstra, A. and Janssen, H. L. (2010). Dendritic cells in chronic viral hepatitis B and C: victims or guardian angels? Gut. 59: 115-125.

World Health Organization: http://www.who.int/mediacentre/factsheets/fs164/en/.

World Health Organization (1999). Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. J. Viral Hepat. 6: 35-47.

Yang, S., Vervaert, C. E., Burch, J., Jr., Grichnik, J., Seigler, H. F. and Darrow, T. L. (1999). Murine dendritic cells transfected with human GP100 elicit both antigen-specific CD8(+) and CD4(+) T-cell responses and are more effective than DNA vaccines at generating anti-tumor immunity. Int. J. Cancer. 83: 532-540.

Yao, N., Reichert, P., Taremi, S. S., Prosise, W. W. and Weber, P. C. (1999). Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease-helicase. Structure. 7: 1353-1363.

Yasui, K., Wakita, T., Tsukiyama-Kohara, K., Funahashi, S. I., Ichikawa, M., Kajita, T., Moradpour, D., Wands, J. R. and Kohara, M. (1998). The native form and maturation process of hepatitis C virus core protein. J. Virol. 72: 6048-6055.

Yewdell, J. W., Norbury, C. C. and Bennink, J. R. (1999). Mechanisms of exogenous antigen presentation by MHC class I molecules in vitro and in vivo: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants, and vaccines. Adv. Immunol. 73: 1-77.

Yu, H., Babiuk, L. A. and van Drunen Littel-van den Hurk. (2004). Priming with CpG-enriched plasmid and boosting with protein formulated with CpG oligodeoxynucleotides and Quil A induces strong cellular and humoral immune responses to hepatitis C virus NS3. J. Gen. Virol. 85: 1533-1543.

Zanoni, I. and Granucci, F. (2010). Regulation of antigen uptake, migration, and lifespan of dendritic cell by Toll-like receptors. J. Mol. Med. (Berl). 88: 873-880.

Zhang, D., Zhang, G., Hayden, M. S., Greenblatt, M. B., Bussey, C., Flavell, R. A. and Ghosh, S. (2004). A toll-like receptor that prevents infection by uropathogenic bacteria. Science. 303: 1522-1526.

Zheng, R., Cohen, P. A., Paustian, C. A., Johnson, T. D., Lee, W. T., Shu, S. and Koski, G. K. (2008). Paired Toll-like receptor agonists enhance vaccine therapy through induction of interleukin-12. Cancer Res. 68: 4045-4049.

Zhu, Q., Egelston, C., Vivekanandhan, A., Uematsu, S., Akira, S., Klinman, D. M., Belyakov, I. M. and Berzofsky, J. A. (2008). Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines. Proc. Natl. Acad. Sci. U.S.A. 105: 16260-16265.

Zhu, Q., Egelston, C., Gagnon, S., Sui, Y., Belyakov, I. M., Klinman, D. M. and Berzofsky, J. A. (2010). Using 3 TLR ligands as a combination adjuvant induces qualitative changes in T cell responses needed for antiviral protection in mice. J. Clin. Invest. 120: 607-616.

7.5 Acknowledgments

Last, but not least I would like to thank all the people who contributed to this work either directly or indirectly, co-workers, colleagues and friends. Without all of you, my work would not have been the same!

In particular, I would like to thank:

Prof. Dr. Dunja Bruder for initiating my challenging and exciting PhD project and for providing confidence, although I am “only” nutritionist. I am very grateful for her scientific input, her optimism, the great ideas and advice. Thank you for being the most open-hearted supervisor one could wish for and that I always found a sympathetic ear whenever needed.

Prof. Dr. Jürgen Wehland for the warm welcome and the opportunity to work in his department Cell Biology.

Prof. Dr. Stefan Dübel for being my mentor at the TU Braunschweig and Prof. Dr. Michael Steinert for being member of my examination committee.

Prof. Dr. Thomas Pietschmann, Prof. Dr. Michael Ott and Dr. Eike Steinmann for being members of my thesis committee, for fruitful discussions and new ideas needed to pursue my project.

All the former and present members of the Immune Regulation Group for all the good times at work and beyond and for being not only colleagues, but also friends! Thank you to Marcus for co-supervising me, for inspiring advice and the help I can always count on. Thank you to Sabine for being “so schlau”, sharing the lab places, and always giving me the support to stick it out. Thank you to Andrea for the “lab dance”, “riding the bears” in Berlin and that I always can build on you, also beyond the work. Thank you to Andreas for being the living example of the “*lag-phase*”. Thank you to Harro for the persistent teasing and the pleasure for me to fight back. Thank you to Silvia for being the busy bee and organizing all the things needed in the daily routine. Thank you to Kathleen, Priya, Jule, Carina and Lisa.

Christian Erck for being a great support, especially in all the things concerning antibodies and ugly proteins as well as for being "your 30 %” at the HZI. Thank you to Marco van Ham for the assistance in binding analyses and the “Koninginnedag”. Thank you to Carsten Schmidt from Thermo Fisher Scientific for discussing all my problems with protein conjugation and giving me the crucial hints.

Prof. Dr. Carlos Guzman for the opportunity to perform the BPPcysMPEG project. Thank you to Thomas and Kai for being on hand with help and advice. Thank you to Angelika for the “animal house” support. Thank you to Elli for teaching me all the practical things and the heated discussions concerning job-related and private things.

Ein großer Dank gilt meiner Familie, insbesondere meinen Eltern Udo und Ulrike Schiller und meiner Schwester Jana: Ohne Euer offenes Ohr, eure Hilfe und Liebe würde ich heute nicht dort stehen, wo ich stehe.

Zuletzt möchte ich mich bei meinem Ehemann Marc bedanken - für sein Verständnis, seine unglaubliche Unterstützung und seinen Zuspruch, vor allem immer dann, wenn es besonders nötig war. Danke für deine endlose Liebe! Du bist mein Fels in der Brandung!

7.6 Curriculum vitae

PERSONAL INFORMATION

Full name	Julia Volckmar geb. Schiller
Date of birth	June 28th, 1983
Place of birth	Braunschweig
Citizenship	German
Contact	julia.volckmar@helmholtz-hzi.de

EDUCATION

01/2008 - 09/2011	PhD student at the Helmholtz Centre for Infection Research Supervisor: Prof. Dr. D. Bruder (Immune Regulation Group) *"Characterizing the potential of DEC-205-mediated antigen delivery to dendritic cells as a tool to induce adaptive immunity against hepatitis C virus infection"* Project in the Helmholtz Alliance on Immunotherapy of Cancer
10/2002 - 12/2007	Studies in Nutrition Science at the Martin-Luther-University Halle-Wittenberg, Germany Certificate: Diplom-Ernährungswissenschaftlerin (final mark 1.3) Diploma thesis at the Martin-Luther-University Halle-Wittenberg, Germany Supervisor: Prof. Dr. K. Eder (Department of Nutrition Physiology) *“Untersuchung der Regulation des Maus-Insig-1-Genes durch den Transkriptionsfaktor PPARα mit Hilfe eines Luciferase-Reportergen-Assays”*
08/1995 - 06/2002	High school in Gifhorn, (Europaschule Humboldt-Gymnasium Gifhorn), Germany Certificate: German Abitur (final mark 2.1)
08/1993 - 08/1995	Middle school in Gifhorn, (Orientierungsstufe Erich-Kästner Gifhorn), Germany Certificate: High School Entrance Diploma
08/1989 - 08/1993	Primary school in Gifhorn, (Wilhelm-Busch Schule Gifhorn), Germany